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SMALL TALK IN THE TUMOR MICROENVIRONMENT – THE CONTRIBUTION OF FIBROBLASTS AND ENDOTHELIAL CELLS TO THE MALIGNANT PHENOTYPE

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**Karolinska
Institutet**

Stockholm 2012

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ISBN 978-91-7457-601-6

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POPULÄRVETENSKAPLIG SAMMANFATTNING

På senare år har man börjat betrakta cancer som ett organ. Precis som vanliga organ i kroppen byggs tumörer upp av flera olika celltyper t.ex. stödjeceller kallade fibroblaster samt blodkärl. Alla de olika celltyperna i tumören kommunicerar med varandra genom signaleringsmolekyler.

Målinriktade farmakologiska behandlingar har på senare tid blivit introducerade som en del av behandlingen av vissa former av cancer. Förhoppningen är att man kan reducera biverkningarna av de nuvarande behandlingarna genom att behandla flera komponenter och signaleringsvägar samtidigt. Denna avhandling har studerat cancer-associerade fibroblaster (CAF) och blodkärl i tumörer för att identifiera mekanismer som skulle kunna vara målet för nya målinriktade behandlingar.

Vi har studerat vad som händer när en speciell form av platelet-derived growth factor (PDGF), PDGF-CC är uttryckt av tumörcellerna. PDGF-CC kan rekrytera CAF till tumören och dessa i sin tur påverkar blodkärlsbildningen så att nya blodkärl bildas, vilket gör att tumören kan växa snabbare. Vi har också sett att när PDGF-CC uttrycks av tumörcellerna i bröstcancer korrelerar det med en kortare överlevnad. Antikroppar kan användas för att blockera signalerna som proteinerna medierar. Vi har utvecklat en sådan antikropp mot PDGF-CC. I en musmodell av bröstcancer växer tumörer som behandlas med denna antikropp långsammare.

I två andra studier har vi studerat vad som händer om man reducerar uttrycket av två receptorer på cellerna som bygger upp blodkärlen, endoglin och Activin Receptor-Like Kinase 1 (ALK1). Tumörer kan anpassa sig till om man tar bort endoglin. Dessa tumörer ger dock upphov till fler metastaser. Om man behandlar tumörerna samtidigt med en annan blodkärlshämmare så utvecklas inte resistens, således skulle det vara intressant att kombinera behandling av dessa två olika signaler samtidigt. Reducerar vi ALK1 istället resulterar det i mindre tumörer med färre blodkärl vilket vi föreslår som en ny målinriktad behandling av cancer.

ABSTRACT

Cancer is the result of aberrant cells developing into tumor cells that act in concert with the micro- and macroenvironment like any other organ in our bodies. The cells and molecules of the tumor microenvironment are important contributors to the tumorigenic process. However, we are still far from understanding the full complexity and intricate molecular interactions that affect the process.

We show that platelet-derived growth factor (PDGF)-CC, expressed by tumor cells, recruit cancer-associated fibroblasts (CAFs) to the tumor microenvironment resulting in an accelerated tumor growth rate. Among the CAFs, we identify three different subclasses by their variable expression of fibroblast specific protein (FSP)-1 and PDGF receptor (PDGFR)- α . Two of these subclasses express osteopontin (OPN), which is responsible for the increased tumor growth rate. In subsequent studies, we reveal that PDGF-CC expression is high in breast cancer and that PDGF-CC presence in epithelial cells is an independent prognostic marker for patient survival in a large cohort of patients. We also developed a monoclonal antibody for PDGF-CC, which as a monotherapy reduces tumor growth rate proving the *in vivo* efficacy of targeting PDGF-CC.

Transforming growth factor (TGF)- β family members have been attributed complex and contradictory effects on angiogenesis. We have studied the effect of ablation of endoglin or activin receptor-like kinase 1 (ALK1) in tumor models. Targeting endoglin results in adaptation and no effect on tumor growth or vessel density. We do however detect increased metastatic seeding as a consequence of endothelial to mesenchymal transition (EndMT). Endoglin deficiency prolongs the tumor sensitivity to antiangiogenic therapy that otherwise induce resistance. However, pharmacological and genetic targeting of ALK1 results in reduction of tumor formation and burden due to decrease in the vessel density. Molecular characterization reveals that the combination of TGF- β and bone morphogenic protein (BMP)-9 is responsible for inducing activation of endothelial cells and angiogenesis.

LIST OF ABBREVIATIONS

ALK1	Activin receptor-like kinase 1
BM	Basement membrane
BMDC	Bone marrow derived cell
BMP	Bone morphogenic protein
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial to mesenchymal transition
ER	Estrogen receptor
FAP	Fibroblast-activated protein
FGF	Fibroblast growth factor
FSP-1	Fibroblast specific protein-1
HGF	Hepatocyte growth factor
Hh	Hedgehog
HHT	Hereditary haemorrhagic telangiectasia
ID	Inhibitor of DNA binding
IFP	Interstitial fluid pressure
IL1RL1	Interleukin 1 receptor like 1
LLC	Lewis lung carcinoma
MEF	Mouse embryonic fibroblast
MMP	Matrix metalloproteinases
MSC	Mesenchymal stem cell
NCAM	Neural cell adhesion molecule
OPN	Osteopontin
PAI-1	Plasminogen activator inhibitor type 1
PDGF/PDGFR	Platelet-derived growth factor/PDGF receptor
PR	Progesterone receptor
SDF-1	Stromal cell-derived factor-1
SMA	Smooth muscle actin
TGF- β	Transforming growth factor- β
VEGF	Vascular endothelial growth factor

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

- I. **Anderberg, C.**, Li, H., Fredriksson, L., Andrae, J., Betsholtz, C., Li, X., Eriksson, U., and Pietras, K. (2009). Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts. *Cancer Res* 69, 369-378.
- II. Li, H*, Kristiansen, G*, **Anderberg, C.**, Scott, A., Neville, M., Stiehl, D., Pietras, K., Moch, H., and Eriksson, U. High epithelial PDGF-CC expression predicts clinical outcome in breast cancer. *Manuscript*
* Equal contribution
- III. **Anderberg, C***, Cunha, S. I*, Zhai, Z*, Pardali, E., Cortez, E., Johnson, J., Franco, M., Páez-Ribes, M., Cordiner, R., Fuxe, J., Goumans, M. J., Casanovas, O., ten Dijke, P., Arthur, H., and Pietras, K. Adaptation to impaired tumor angiogenesis in endoglin-deficient mice is paralleled by a weakened endothelial barrier to metastatic dissemination. *Manuscript*
* Equal contribution, alphabetical order
- IV. Cunha, S. I., Pardali, E., Thorikay, M., **Anderberg, C.**, Hawinkels, L., Goumans, M. J., Sehra, J., Heldin, C. H., ten Dijke, P., and Pietras, K. (2010). Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *J Exp Med* 207, 85-100.

Other publications not included in the thesis:

Anderberg, C., and Pietras, K. (2009). On the origin of cancer-associated fibroblasts. *Cell Cycle* 8, 1461-1462.

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1 INTRODUCTION

Cancer is one of the most common diseases and the lifetime risk of being affected is around 40% in the US (ACS, 2007) and Sweden (Åberg, 2010). Despite advances in both detection and treatment, the mortality rate in cancer is approximately 20% (ACS, 2007). The sites most often affected include breast in women and prostate in men followed by skin and colon cancer for both genders. In general, tumors arise in 80% of the cases from epithelial tissues whereas 19% are leukemias/lymphomas, which are derived from the hematopoietic cells. Around 1% of the tumors are sarcomas arising in the connective tissue (Weinberg, 2007).

Historically, cancer research has been focused on the tumor cells *per se* exhibiting traits of loss control over cell division with very little attention put on the other cells that are present in the vicinity. However, pathologists early recognized that some tumors also contained an abundance of other cell types and extracellular matrix (ECM) and there were early ideas about the importance of the other components of the tumor. Already in 1971, Judah Folkman articulated the idea that inhibiting tumor angiogenesis could inhibit tumor growth (Folkman, 1971; Kerbel, 1997). In the 1980's, histological studies suggested striking similarities between tumors and wound healing, leading up to the proposition that the tumor is able to abuse or exaggerate normal physiological processes (Dvorak, 1986). However, only a minute part of cancer research paid attention to those areas.

In an attempt to summarize all the conclusions from decades of previous research within the cancer field, Hanahan and Weinberg wrote their now classical review condensing cancer into six functional capabilities that needed to be acquired in order for a tumor to form (Hanahan and Weinberg, 2000).

These capabilities include:

- self-sufficiency in growth signals
- insensitivity to anti-growth signals
- tissue invasion and metastasis
- limitless replicative potential
- sustained angiogenesis
- evasion of apoptosis

Although not explicitly mentioned, the tumor cells themselves are considered to acquire all the traits described.

The emerging view of cancer today is that it is a disease built up by several cell types, all working in conjunction as any other organ in our body (Radisky et al., 2001), and that this organ also has the ability to communicate with the rest of the organism turning cancer into a systemic disease. The tumor microenvironment is composed of the cancer cells, endothelial cells and pericytes forming the tumor vasculature, several types of immune cells, CAFs and the ECM, as well as circulating cells recruited from the bone marrow (Joyce, 2005; Pietras and Ostman, 2010). The six hallmarks of cancer do not

necessarily need to be acquired by the tumor cells themselves (Pietras and Ostman, 2010), but can rather be the result of paracrine crosstalk with the tumor stroma. Recently, the follow-up of the Hallmarks of Cancer was published. Two new emerging hallmarks were included *i.e.* deregulation of cellular energetics and avoiding immune destruction. However, these are not fully characterized and generalized yet, hence the label as emerging. They have also included two consequential characteristics that facilitate the achievement of hallmarks, tumor-promoting inflammation and genome instability and mutation. It is also emphasized that the tumor microenvironment contributes to the tumor development and characteristics (Hanahan and Weinberg, 2011).

1.1 THE TUMOR MICROENVIRONMENT

The tumor microenvironment was early on considered to be unchanged by the tumorigenic process. However, studies have shown that the microenvironment is not unchanged during the development of a tumor; it evolves together with the tumor cells (Polyak et al., 2009). The microenvironment can also be the driver of tumor development. Early experiments where the mammary fat pad was cleared of epithelial cells, then irradiated followed by re-transplantation of epithelia reported that the tumor number was significantly increased compared to non-irradiated hosts (Barcellos-Hoff and Ravani, 2000). Similar results were obtained when irradiated fibroblasts were grafted together with weakly tumorigenic pancreatic cells (Ohuchida et al., 2004). In addition, injection of tumor cells in *p53* knockout mice displayed enhanced tumor growth rate (Kiaris et al., 2005). The non-malignant cells and components of the tumor microenvironment have implications for cancer prevention, diagnosis and treatment (Polyak et al., 2009).

The components of the tumor microenvironment will be discussed briefly below. Angiogenesis and fibroblasts will be discussed in more detail, as they are the foundation of the work this thesis is based on.

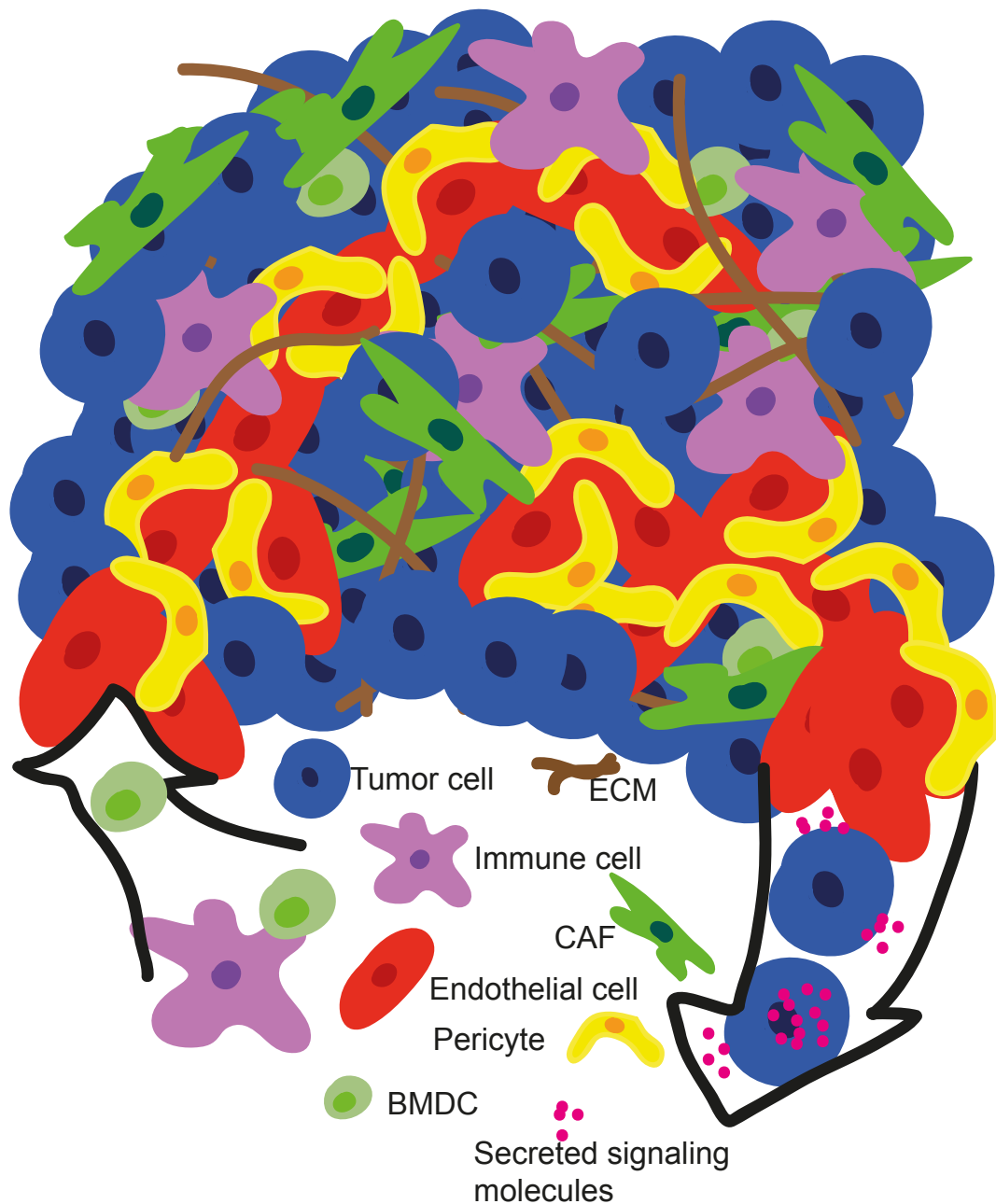


Figure 1. The components of the tumor micro- and macroenvironment.

The tumor microenvironment contains not only tumor cells but also ECM, immune cells, CAFs, bone marrow derived cells (BMDC) and the endothelial cells and pericytes building up the vasculature. The tumor also communicates over distance recruiting cells, in addition to releasing paracrine factors with systemic action and seeding tumor cells for metastasis.

1.1.1 ECM

The ECM consists of a complex mix of glycoproteins, proteoglycans, cytokines and growth factors (Aumailley and Gayraud, 1998; Bissell et al., 1982) and provides a scaffold for the tissue in order to provide important contextual information. Fibroblasts and epithelial cells produce the ECM in collaboration (Bissell and Radisky, 2001). The

basement membrane (BM) is a specialized type of ECM lining the epithelium and vasculature (Radisky et al., 2001). As the requirements of tissues are different, the ECM composition varies accordingly in the healthy situation (Streuli, 1999; Tsai, 1998). The ECM in tumors is abnormal and may contain components such as tenascin, fibronectin and alternative isoforms of laminins (Lohi et al., 1998; Wenk et al., 2000), which has implications for the balance of proliferation and apoptosis in the tumor (Ramos et al., 1998; Schoenwaelder and Burridge, 1999; Sethi et al., 1999). The ECM in tumors resembles that of a healing wound (Dvorak, 1986).

1.1.2 Inflammation

Several forms of tumors are associated with chronic inflammation (Joyce, 2005; Virchow, 1858). The tumor cells themselves, but also other cell types, e.g. CAFs, produce a variety of different chemokines and cytokines that attract components of the immune system. The tumor microenvironment contains neutrophils, dendritic cells, eosinophils, mast cells, macrophages and lymphocytes. These cells are able to produce cytokines, cytotoxic agents, membrane disruptive agents, as well as a plethora of different proteases, angiogenic and lymphangiogenic growth factors (Kuper et al., 2000; Mantovani et al., 2008; Schoppmann et al., 2002; Wahl and Kleinman, 1998). The factors that these immune cells produce affect both tumor cells and the tumor micro- and macroenvironment. However, the contribution of the immune system component will not be discussed in further detail.

1.1.3 The tumor macroenvironment

As pointed out earlier, a tumor also has systemic effects. Inflammatory cells are recruited from a distance. In addition fibroblasts (discussed below) and to some extent probably endothelial cells (Rafii et al., 2002) may also be recruited to the tumor microenvironment. These cells are most likely derived from the bone marrow. Experimental evidence points to that this is an active recruitment by the tumor that produces factors such as TGF- β and OPN that mobilize and recruit cells from a distance to the tumor (McAllister et al., 2008; Yang et al., 2008).

Already in 1889 Paget pointed out that tumors metastasized to specific, not random, places depending on the primary tumor (Paget, 1989). The spread of tumors through the circulation may hold part of the explanation because of the physical limitations it presents. However, recent investigations have provided evidence that the site of metastasis may be influenced by the primary tumor to create a welcoming microenvironment through systemic effects. As proof of principle that the primary tumor influences the site of metastasis Lewis Lung carcinoma (LLC) bearing mice were injected with medium from B16 melanoma cells and this was enough to redirect metastases to sites where they are commonly found for the melanoma model (Kaplan et al., 2005). There are data indicating that BMDC are present at the metastatic site before arrival of the tumor cells (Wels et al., 2008). The systemic stimuli, when it comes to preparing the metastatic site, and the primary tumor for that matter, does not necessarily need to be circulating proteins, microvesicles may also be an important factor (Peinado et al., 2011). Remodeling of the metastatic site through lysyl oxidase,

that crosslinks proteins of the ECM, secreted by the tumor may also be an event important for a welcoming microenvironment (Psaila and Lyden, 2009).

Certain tumors have the ability to influence growth of otherwise very slow growing malignant cells that not consistently form tumors in mice. Using this system, OPN was identified as the main stimulus for the mobilization of BMDC that enabled the growth of the slow growing malignancy (McAllister et al., 2008). Follow-up experiments implicated a desmoplastic stroma, created by fibroblasts at the recipient tumor site, as responsible for the increased tumor growth. The fibroblasts were activated by the recruited BMDC (Elkabets et al., 2011). This may also have implications for metastasis as the systemic stimuli also influenced growth of metastases (McAllister et al., 2008).

1.2 FIBROBLASTS

Fibroblasts are in general a very heterogeneous population, which originally was, and still to a large extent is, identified by the morphological appearance as spindle-shaped elongated cells within the ECM and lack of markers suggesting other cell lineages (Kalluri and Zeisberg, 2006). There is a large problem with identifying fibroblasts with molecular markers as no marker labels all fibroblasts and many markers used are not exclusive for fibroblasts (Kalluri and Zeisberg, 2006; Sugimoto et al., 2006).

Fibroblasts are important producers of ECM components as well as highly engaged in the remodeling of the scaffold through secretion of proteases. Fibroblast and epithelial interactions are important during development for example in breast, skin and lung (Hogan and Yingling, 1998; Simian et al., 2001; Sorrell and Caplan, 2009). In addition, fibroblasts are instrumental in wound healing. Following fibrin clot formation fibroblasts migrate into the clot towards growth factors such as fibroblast growth factor (FGF), TGF- β and PDGF. The two latter converts them into activated fibroblasts, which apply contractile force closing the wound. The activated fibroblasts go through apoptosis during the end stages of wound healing (Darby and Hewitson, 2007). If fibroblasts are activated improperly or activation not terminated at the correct time point, fibrosis will be induced. TGF- β signaling, especially non-canonical, has recently been suggested as an anti-fibrosis treatment (Rosenbloom et al., 2010).

1.2.1 Fibroblasts in tumors

The CAF, or sometimes tumor-associated fibroblast, is the most common cell type in the tumor stroma and has prominent influences on the tumor development and progression. The widest definition of a CAF, that will be used here, is all fibroblasts within the tumor stroma, regardless of the activation status as shown by expression of myofibroblastic markers (Micke and Ostman, 2004). As their normal counterparts, CAFs come in many different flavours expressing a variety of different molecular markers. These identified subpopulations of CAFs express markers such as FSP-1 (also S100A4), fibroblast-activated protein (FAP), α -smooth muscle actin (SMA), vimentin and PDGF receptor (PDGFR)- α and - β (Micke and Ostman, 2004; Ostman and Augsten, 2009; Sugimoto et al., 2006). Some of these markers also label pericytes, smooth muscle cells and macrophages. Hitherto, no marker has been demonstrated to

label all CAF subpopulations. This diversity and inconsistency of marker expression underlines the heterogeneity of CAFs with regard to their functional properties within the tumor microenvironment, just as the normal tissue fibroblasts.

1.2.1.1 Origin of CAFs

In coherence with their heterogeneity, CAFs have been proposed to originate from several different sources. All these studies have been performed in mouse models. Resident tissue fibroblasts have been shown to be a source of CAFs (Micke and Ostman, 2004). Transdifferentiation of epithelial and endothelial cells into mesenchymal cells is another recruitment path. Epithelial to mesenchymal transition (EMT) as a source of fibroblasts has been shown in fibrosis (Iwano et al., 2002) but also in tumors where similar genetic changes have been detected in tumor cells and CAFs (Ellsworth et al., 2004; Fukino et al., 2004; Kurose et al., 2002; Petersen et al., 2003; Tuhkanen et al., 2004). Zeisberg and colleagues have shown that endothelial cells in two different tumor models down regulate CD31 concomitant with up regulation of FSP-1 *in vivo*. Similar changes were recorded *in vitro* following TGF- β stimulation of primary endothelial cells (Zeisberg et al., 2007). Less established is transdifferentiation of other cells in the tumor stroma, including vascular mural cells and adipocytes, which may be an explanation for the appearance of pericyte markers on CAFs (Anderberg and Pietras, 2009; Ronnov-Jessen et al., 1995; Xing et al., 2010). The CAFs can also be recruited systemically in the form of mesenchymal stem cells (MSC) and bone marrow precursor cells. This has been shown through transplantation experiments where labeled bone marrow cells are detected in the tumor stroma as CAFs (Direkze et al., 2004; Ishii et al., 2005; Sangai et al., 2005). These means of recruitment of CAFs are not mutually exclusive and several processes are most likely involved in contributing to the diverse CAF populations seen in experimental tumors. The origin of CAFs in human tumors has not been an area of research that has been intensely investigated.

1.2.1.2 The contribution of CAFs to the malignant phenotype

Early experiments *in vitro* and in mouse models have confirmed the importance of CAFs as active players in the tumorigenic process. These experiments indicated that CAFs are functionally different in this aspect from normal fibroblasts, which strives to normalize the malignancy (Barcellos-Hoff and Ravani, 2000; Hayward et al., 2001; Olumi et al., 1999).

CAFs produce several growth factors from a number of families that act in an autocrine or paracrine fashion in the tumor microenvironment. These include the FGF family, the insulin growth factor family, the epithelial growth factor family, hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1/CXCL12), CXCL14, matrix metalloproteinases (MMPs) and the TGF- β family. These factors act as proliferative and morphogenic stimuli for the tumor and may also affect other properties such as angiogenesis, inflammation and remodeling of the ECM releasing for example sequestered growth factors (Augsten et al., 2009; Bhowmick and Moses, 2005; Bhowmick et al., 2004b; Orimo and Weinberg, 2006; Thijssen et al., 2004; Xing et al., 2010). Early experiments using human derived

fibroblasts overexpressing HGF together with human mammary epithelial cells transplanted into cleared fat pads of mice yielded invasive carcinomas, whereas fibroblasts not expressing HGF failed to induce carcinoma (Kuperwasser et al., 2004). HGF has mitogenic effects through signaling via its receptor cMet, commonly found on epithelial cells, in addition to migratory and angiogenic effects (Jiang et al., 2005). Another set of experiments investigated the molecular mechanism in more detail. Human CAFs enhanced tumor growth in mice when co-injected with breast carcinoma cells compared to normal human fibroblasts from breast. This was shown to be due to CAF derived SDF-1 which recruited endothelial progenitor cells enhancing angiogenesis but SDF-1 also have a direct effect on tumor cell proliferation (Orimo et al., 2005). CAFs produce several matrix-remodeling proteins, such as MMPs, and have in an *in vitro* model been shown to be able to lead the invasion of carcinoma cells that lack ability to remodel matrix themselves (Gaggioli et al., 2007).

Gene expression profiling studies indicate a high degree of heterogeneity within the CAF population. Some of the differences are likely explained by the use of tumors of various origin, different methods and also the use of different criteria in defining the normal fibroblastic compartment used for comparison (Micke and Ostman, 2005). For example, the normal fibroblasts that the CAFs gene expression profile is compared to may be derived from the same patient at a certain distance from the tumor (Micke et al., 2005) or from unrelated normal tissue (Allinen et al., 2004; Fukushima et al., 2004). The method of separating the CAFs from the tumor cells is also different, some used laser microdissection (Fukushima et al., 2004; Micke et al., 2005) whereas others chose antibody bead based cell sorting (Allinen et al., 2004).

Orimo and colleagues have provided important information regarding the development of the phenotype of CAF isolated from breast carcinomas. The isolated CAFs, although heterogeneous, all stimulated tumor growth when co-injected with breast tumor cells subcutaneously compared to normal fibroblasts isolated from a healthy patient. Interestingly, fibroblasts isolated 2 cm away from the tumor also displayed similar changes as the isolated CAFs, albeit at considerably lower levels. The CAF co-injected tumors displayed increased angiogenesis due to increased expression of SDF-1 (Orimo et al., 2005). In a subsequent study, autocrine SDF-1 and TGF- β were identified as mediators of maintenance of the CAF phenotype (Kojima et al., 2010).

1.2.1.3 Therapeutic implications of CAFs

The CAFs in tumors poses an interesting opportunity for targeted therapies. PDGF inhibitors are already used in the clinic, although usually for other implications that is discussed below. One of the earliest targeted therapies directed towards CAFs was MMP inhibitors. They have been non-successful so far in clinical trails and display significant side effects (Bissett et al., 2005; Leighl et al., 2005; Miller et al., 2004; Rosenbaum et al., 2005). Considering that the attention that CAFs has been given in research is far less than that for angiogenesis, it is not surprising that not more targeted therapies are available. However with the increasing attention and knowledge the field is attracting, most likely novel therapies will be developed.

PDGFRs are commonly found in the tumor stroma. Tyrosine kinase inhibitors applied in a cervical cancer model and a colon cancer, where CAFs have been shown to express PDGFRs, significantly reduced tumor growth rate (Kitadai et al., 2006; Pietras et al., 2008). Although suggestive of that this is part of the clinical effect of tyrosine kinase inhibitors in clinical trials, however, the evidence from the human situation is lacking. A recent publication suggests that targeting fibroblasts expressing FAP using a DNA vaccine strategy changed the immune response in the tumor and also sensitized them to chemotherapy (Liao et al., 2009). Another recent publication suggests targeting of paracrine hedgehog (Hh) signaling to the stroma by neutralizing antibodies or small molecule inhibitors. In short, Hh ligands were identified in a subset of human cancers. However, no activity of Hh signaling could be identified in human cancer cell lines but Hh inhibitors reduced tumor growth rate *in vivo*. Through co-injection experiments using MEFs deficient in Hh signaling they show that these fibroblasts recapitulate the growth inhibitory phenotype suggesting that they are the target of the Hh inhibitors *in vivo* (Yauch et al., 2008).

A gene signature that predicts clinical outcome was identified using laser micro-dissection of human breast cancer stroma (Finak et al., 2008). In another study, using a different approach where the stroma signature was extracted from the gene expression profile of tumor tissue, they were not able to predict clinical outcome, but instead identified patients less likely to benefit from neo-adjuvant therapy (Farmer et al., 2009). The major stromal component in breast tumors is CAFs, indicating that these results most likely reflect the CAF population in the tumors.

1.2.2 The PDGF system

The PDGFs are a family of four gene products forming five isoforms of dimeric disulphide bonded growth factors (Fredriksson et al., 2004a). They signal through two different tyrosine kinase receptors *i.e.* the PDGFR- α and PDGFR- β which homo- or heterodimerize to cross-phosphorylate each other (Claesson-Welsh et al., 1989; Yarden et al., 1986). The PDGFR- α interacts with all isoforms except PDGF-DD while PDGFR- β only binds to PDGF-B and -D chains with high affinity. PDGF-AB, -BB and -CC interacts with the PDGFR- $\alpha\beta$ heterodimer (Fredriksson et al., 2004b). Conflicting results have been reported with regard to the PDGF-DD activation of PDGFR- $\alpha\beta$ heteromere (Bergsten et al., 2001; LaRochelle et al., 2001). The PDGFR signaling activates several intracellular downstream signaling pathways in a cell type specific manner (Rosenkranz and Kazlauskas, 1999).

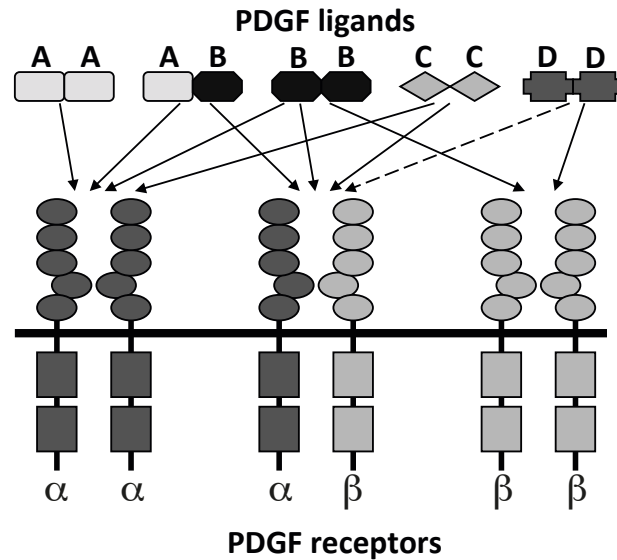


Figure 2. The structure of the PDGFR dimers and binding pattern of the different PDGF ligands to the receptors.

The classical PDGFs, PDGF-AA, -AB and -BB, are secreted as activated proteins (Ostman et al., 1992) whereas the novel PDGFs, PDGF-CC and -DD need to be proteolytically activated (Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2000b). Tissue plasminogen activator and urokinase-type plasminogen activator have been identified as the probable proteolytic enzymes for PDGF-CC and -DD respectively (Fredriksson et al., 2004b; Ustach and Kim, 2005). Recently, matriptase was described as a new protease able to activate PDGF-CC (Hurst Jr et al., 2011). In addition, both PDGF-CC and -DD can be activated by plasmin, but due to the wide substrate specificity it is most likely not the physiologically relevant enzyme (Fredriksson et al., 2004b).

1.2.2.1 Functions of PDGFs

The PDGFs have been shown to be important during development but there is limited information about their normal physiological functions in the adult situation (Andrae et al., 2008). Genetic studies in mice have revealed important information regarding the function of members of the PDGF signaling system during development. Knockout mice of *pdgfr-b*, *pdgfr- α* and *pdgfr- β* are all embryonically lethal (Leveen et al., 1994; Soriano, 1994; Soriano, 1997). The *pdgfr-a* knockout is lethal but time of lethality ranges from embryonic stage to 60 days postnatal, most likely depending on strain background (Bostrom et al., 1996). The *pdgfr-c* knockout is viable, at least in some backgrounds (Ding et al., 2004). The *pdgfr-d* knockout is yet to be reported. In short, the combination of *pdgfr-a* and *pdgfr-b* does not fully recapitulate the phenotype of *pdgfr- α* knockdown, suggesting that *pdgfr-c* also contributes to embryonic development (Betsholtz et al., 2001; Ding et al., 2004). These knockouts display cleft palate, spina bifida and skin, lung and brain abnormalities with varying overlap (Andrae et al., 2008; Betsholtz, 2004). The *pdgfr-b* and *pdgfr- β* knockouts are very similar. They develop

cardiovascular complications and histological findings include several vascular abnormalities most likely due to inability to recruit mural cells (Betsholtz et al., 2001; Lindahl et al., 1997; Soriano, 1994).

The PDGFs are involved in several pathological conditions including cancer, vascular diseases and fibrosis (Andrae et al., 2008). This is in line with the early notion that the PDGFs are a major mitogen for cells of mesenchymal origin, including vessel mural cells and fibroblasts (Heldin and Westermark, 1999).

1.2.2.2 PDGF-CC

PDGF-CC expression has been demonstrated to induce fibrosis in a number of different organs such as liver, kidney and heart (Campbell et al., 2005; Eitner et al., 2008; Ponten et al., 2003). The phenotype of knock out of *pdgf-c* in mice has shown that PDGF-CC is important for palate formation, renal cortex formation and the lung, skeletal and vascular development. Spina bifida is also seen in the knockouts (Ding et al., 2004). Depending on the mouse background, the phenotype is more or less severe. C57Bl/6 *pdgf-c* knockout mice are viable but numbers of surviving pups are slightly reduced compared to the expected Mendelian ratio. PDGF-CC has also been suggested to stimulate angiogenesis in different systems (Cao et al., 2002; Li et al., 2005) and in addition be implicated in regulation of the blood brain barrier integrity (Su et al., 2008).

1.2.2.3 PDGFs in the tumor microenvironment

All PDGF isoforms have been shown, in different carcinomas, to be produced by tumor cells. It is also common that the tumor cells lack expression of the PDGFR implying that the PDGFs produced in the tumor communicate with stromal cells and aid in their recruitment (Heldin and Westermark, 1999; Mücke and Ostman, 2005). Autocrine stimulation or activating mutations of PDGFRs of tumor cells has been described (Ostman, 2004) but will not be discussed further here.

Early research performed has focused on the outcome effect of tumor cell PDGF-BB expression in melanoma on the tumor stromal compartment in tumor initiation and development. These experiments suggested that tumor microenvironment stimulated with PDGF-BB was able to increase stroma formation and angiogenesis (Forsberg et al., 1993). Similar results were obtained when PDGF-BB was expressed in non-tumorigenic keratinocytes. This resulted in induction of an activated stroma and angiogenesis and promoted the keratinocyte conversion into tumorigenic cells (Skobe and Fusenig, 1998). In line with this, Shao and colleagues show that breast cancer cells expressing the classical PDGFs are able to induce a desmoplastic response when injected orthotopically (Shao et al., 2000). Additional information regarding the effect of PDGF-BB and -DD expression was obtained in the B16 melanoma mouse model where these factors increased the pericyte abundance in tumors conferring an increased tumor growth rate (Furuhashi et al., 2004). Giving mechanistic insight into the tumor angiogenesis induced by PDGFs, PDGFR- α signaling was demonstrated to be required for recruitment of host fibroblast secreting VEGF in a fibrosarcoma system devoid of tumor VEGF expression (Dong et al., 2004).

The interstitial fluid pressure (IFP) is elevated in most solid tumors. This occurs due to abnormal vascular and interstitial permeability and capillary pressure. IFP is an important factor for efficient drug delivery into tumors and distribution within tumors as the high IFP may require elevated blood concentration of drugs to achieve the correct dose for the tumor, hence, increasing the toxicity to the rest of the body (Lunt et al., 2008). Inhibition of the PDGFRs results in increased drug delivery to the tumor in several tumor models (Pietras et al., 2001; Pietras et al., 2002; Pietras et al., 2003).

Recently, several studies investigating the significance of PDGFR expression in human cancers have been published. Interestingly, using the PDGFRs as markers, they identify four different subsets of CAFs expressing none, one receptor or both receptors in several different cancers. High stromal fibroblast expression of PDGFR- β in human breast cancer samples was associated with a shorter recurrence free survival and shorter breast cancer specific survival, hence high PDGFR- β expression in fibroblasts display prognostic significance (Paulsson et al., 2009). Stromal PDGFR- β expression in prostate cancer has also been shown to correlate with several characteristics of poor prognosis (Hagglof et al., 2010). A subset of glioblastoma tumors display tumor cell PDGFR- α expression and amplification of the *pdgf-a* gene. Histopathological investigation of PDGFR and activated PDGFR status of tumors from patients participating in a trial, where the tyrosine kinase inhibitor imatinib was used together with chemotherapy, revealed that activated PDGFR- α in tumor cells was associated with reduced survival. However, the addition of imatinib to chemotherapy did not improve survival (Paulsson et al., 2011).

1.2.2.4 PDGF-CC in the tumor microenvironment

In 2001 *pdgf-c* was suggested to be an important mediator of malignancy in the Ewing family tumors (Zwerner and May, 2001). As previously mentioned, PDGF-CC overexpression in liver induces liver fibrosis causing multifocal liver tumors in mice (Campbell et al., 2005). Investigating the phenotype closely revealed an increased proliferation of both hepatic stellate cells and endothelial cells preceding the tumor development in these mice. As imatinib treatment was able to block stromal cell proliferation in this model they suggest this as a possible treatment for human hepatocellular carcinoma with expression of c-kit or PDGFRs (Campbell et al., 2007). PDGFR- α signaling has been shown to be involved in recruiting stromal fibroblasts into lung tumors in a mouse model. However, both PDGF-AA and -CC is present in this system. In addition, the two ligands are present in human lung tumors along with expression of PDGFR- α (Tejada et al., 2006). Further insight into the role of stromal PDGF signaling in tumor development was provided in a genetically engineered mouse model of cervical cancer. The PDGFRs are expressed in the stromal compartment. Inhibiting tyrosine kinases through imatinib treatment reduces tumor growth through actions on angiogenesis by reducing FGF-2 production by CAFs. The ligand most abundantly expressed was *pdgf-c* and high PDGF-CC expression was also noted in human tumor epithelium suggesting that it is the responsible ligand for the observed phenotype (Pietras et al., 2008). CAFs isolated from VEGF treatment responding or resistant tumors reveal that PDGF-CC was expressed by the resistant CAFs and that neutralizing PDGF-CC using antibodies attenuated the sustained angiogenesis provided by the CAFs in the resistant tumors (Crawford et al., 2009). PDGF-CC expression has

also been noted in glioblastoma. Overexpression of PDGF-CC in glioblastoma cells resulted in tumors displaying a normalized vasculature with reduced permeability and vessel diameter together with an increase in pericyte coverage. The use of a VEGF antibody in the PDGF-CC overexpressing tumors had no effect while the therapy has effect in the parental tumor (di Tomaso et al., 2009). Taken together, these experimental results suggest that targeting either the PDGF ligand or receptor may be a successful strategy when these targets are expressed in the human situation.

1.3 ANGIOGENESIS

Angiogenesis is the formation of blood vessels from existing blood vessels, as opposed to vasculogenesis where the vessels are formed *de novo*. The blood vessels are built up by endothelial cells, which are surrounded by BM and mural cells, pericytes or vascular smooth muscle cells. The vascular system is normally quiescent in the adult except for wound healing and during the female reproductive cycle. New studies also suggest that the endothelium have important functions in determining size and regeneration of organs. Through pneumonectomy and hepatectomy experiments in mouse models it has been shown that endothelial derived cues directs epithelial regeneration (Ding et al., 2010; Ding et al., 2011). Angiogenesis is kept quiescent through a delicate balance of pro- and antiangiogenic factors. A number of factors have been implicated in angiogenesis. Important signaling systems include VEGF, FGF, Notch signaling, angiopoietins, PDGF and TGF- β which all have been shown to have important functions during vascular development in mouse and zebra fish model systems (Betsholtz, 2004; Bikfalvi et al., 1997; Ferrara, 2004; Gale and Yancopoulos, 1999; Gridley, 2010; ten Dijke and Arthur, 2007).

1.3.1 Tumor angiogenesis

It is now almost exactly 40 years ago since Folkman published his now famous review articulating old hypotheses that inhibiting angiogenesis in tumors would be a good treatment to inhibit tumor growth (Folkman, 1971). In order for the tumor to grow beyond a few millimeters in size it needs to secure its blood supply. Interference with the intricate balance of angiogenic effector molecules early during the tumorigenic process is imperative to turn on the angiogenic switch and tip the balance in favour of angiogenesis and hence, sustained tumor growth (Hanahan et al., 1996; Hanahan and Folkman, 1996). Prototypical angiogenic inducers in tumors include VEGF and FGF growth factors (Hanahan and Folkman, 1996). The vessels in the tumor are commonly tortuous and display abnormal features with regard to permeability, morphology and composition (Baluk et al., 2005; Bergers and Benjamin, 2003; Hashizume et al., 2000; Hillen and Griffioen, 2007; Jain, 2003).

1.3.1.1 Antiangiogenic therapy

Antiangiogenic therapy has been very successful in preclinical experiments. One of the first experiments showing that inhibition of angiogenic factors reduced tumor growth rate and vessel density was reported in 1993. They treated three different subcutaneously growing tumors with VEGF inhibitory antibodies (Kim et al., 1993). Inhibiting angiogenesis using endostatin instead in three syngeneic tumor mouse

models, it was suggested that angiogenesis inhibitors were not able to induce resistance as treatment was discontinued once the tumor had regressed and re-initiated when the tumor regrew. This was repeated in several cycles until the tumors did not regrow (Boehm et al., 1997). The efficacy of VEGF kinase inhibitors on tumor growth rates in subcutaneous models was reported later (Fong et al., 1999; Wood et al., 2000).

There were high hopes when antiangiogenic therapy was introduced into the clinic in the form of the VEGF monoclonal antibody bevacizumab (Avastin) 2004 (Folkman, 2006). However, the high hopes were not fulfilled with regard to overall survival and a limited effect of a few months increase in progression free survival in renal cell carcinoma and metastatic colon cancer (Escudier et al., 2007; Hurwitz et al., 2004; Motzer et al., 2007). Earlier this year, the United States Food and Drug Administration revoked the approval for use of bevacizumab in breast cancer due to lack of increased overall survival (Twombly, 2011). Evasive resistance most likely brings on the lack of increase in overall survival.

Thorough investigations in preclinical mouse models have implicated several mechanisms of resistance, either inherent or acquired, that may be the explanations to the absent clinical survival benefit to the antiangiogenic treatments (Bergers and Hanahan, 2008; Ebos et al., 2009b). In more detail, glioblastoma tumors have shown a pattern of slowed down tumor growth and reduced vascularity when treated with anti-VEGF antibodies, but the tumor invasive pattern changed and the tumor co-opted normal host vessels (Rubenstein et al., 2000). Induction of other pro-angiogenic factors have also been noted when tumors adapt and acquire resistance to anti-VEGF therapy. Tumor growth was retarded transiently but after a short period the tumors again appeared at equal size. The mechanism behind this induced resistance was hypoxia-induced induction of FGF-2 and other proangiogenic factors (Casanovas et al., 2005).

It is known that pericytes are able to stabilize vessels and that targeting both pericytes and endothelial cells simultaneously is beneficial (Bergers et al., 2003). VEGF therapy has also been shown to increase pericyte coverage of vessels, protecting the vessels for the effect of the antiangiogenic treatment (Bergers et al., 2003; Jain, 2005; Jain and Booth, 2003; Pietras and Hanahan, 2005). In addition, pericyte marker expression changes upon anti VEGF therapy suggestive of induction of changes in the pericytes upon antiangiogenic therapy (Franco et al., 2011a). A study of melanoma in a mouse model showed that some tumors are inherently resistant to antiangiogenic therapy with VEGF inhibitors due to a different vessel structure and high degree of pericyte coverage (Helfrich et al., 2010). Recently, detailed studies have described that pericytes, through integrins, induce a survival signal in endothelial cells involving VEGF and the antiapoptotic factor Bcl-w (Franco et al., 2011b). Another mechanism to restore angiogenesis that has been described is recruitment of BMDC as a result of hypoxia in the tumor (Bergers and Hanahan, 2008). Other cell types, inflammatory cells and CAFs, in the tumor microenvironment can also aid the tumor to overcome anti-VEGF therapy by expression of angiogenic factors (Crawford and Ferrara, 2009; Crawford et al., 2009; Shojaei et al., 2007).

Metastases are a major problem in the clinic and are responsible for about 90% of the cancer related deaths (Steeg and Theodoreescu, 2008). Recent reports have highlighted

the connection between antiangiogenic therapy and enhancement of metastasis. Specifically, both genetic and therapeutic targeting of the VEGF pathway caused tumors displaying enhanced invasion and increased metastasis (Paez-Ribes et al., 2009). In addition, a small molecule kinase inhibitor targeting the VEGF/PDGF receptors caused accelerated metastasis in parallel to an antitumoral effect on a bulk tumor (Ebos et al., 2009a). However, so far there are no reports from the clinic regarding the enhancement of metastasis following resistance to antiangiogenic therapy.

1.3.2 The TGF- β family

TGF- β signaling is portrayed as a dual player during tumorigenesis with generally a tumor suppressive role in the premalignant state and a tumor promoting effect at later stages (Massague, 2008; Tian and Schiemann, 2009). The TGF- β paradox has been investigated for years but we still have a lot to learn about this complex and intricate signaling system.

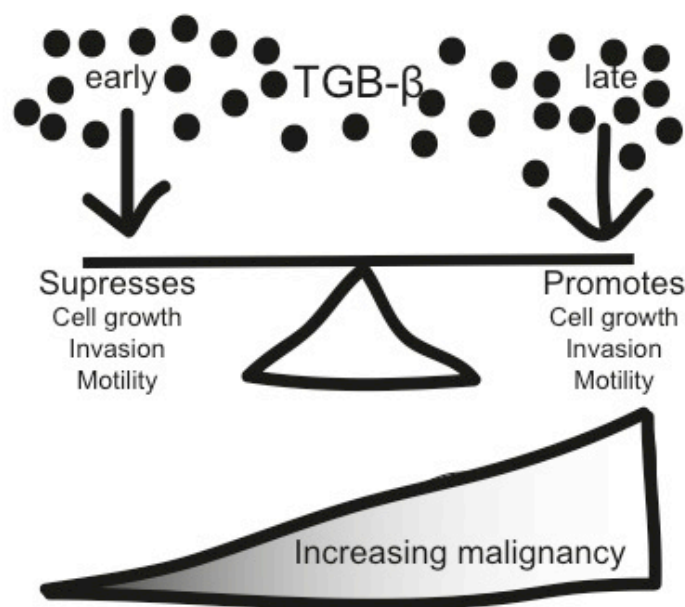


Figure 3. Illustration of the TGF- β paradox.

TGF- β signalling is involved in both tumor suppression and promotion activities.

The TGF- β family has a large number of ligands divided into subclasses comprising of TGF- β s, Activins, Nodal, BMPs, growth and differentiation factors, and muellerian inhibiting substance (Shi and Massague, 2003). The ligands act as dimers signaling through serine/threonine kinase receptors of two different classes, TGF- β type I and type II receptors with seven and five members respectively. There are also three co-receptors sometimes referred to as type III receptors. The dimeric ligand binds to dimers of type II and type I receptors forming a heterotetrameric complex, sometimes including type III receptors in the large signaling complex. The exact order of events varies (Schmierer and Hill, 2007). The type I receptor is phosphorylated by the type II

receptor which causes recruitment of the receptor-regulated SMADs. The type I receptor phosphorylates the receptor-regulated SMAD which then forms a heteromeric complex together with SMAD4. This complex is translocated to the nucleus where it participates in regulation of target genes. Inhibitory SMADs are induced by SMAD signaling and mediate negative feedback on TGF- β signaling (Moustakas and Heldin, 2005). This canonical intracellular signaling pathway has been conserved throughout evolution. In addition there are also other, non-SMAD signaling pathways identified. Cytoplasmic effector molecules that have been identified include Ras, mitogen-activated protein kinases, extracellular-signal-regulated kinases, p38 and c-Jun N-terminal kinases. Most of these downstream signaling proteins were identified *in vitro* by pharmacological inhibitors and the exact link and order of events remains to be identified (Moustakas and Heldin, 2005).

The members of the TGF- β family are important for a number of processes during development, in the adult and in several diseases. The discussion here will focus on the role of the TGF- β family in cancer. The TGF- β family has the ability to impinge upon tumor cells and all cell types in the tumor microenvironment (Tian and Schiemann, 2009). In the premalignant state, TGF- β inhibits malignancy through regulation of proliferation, differentiation, survival, adhesion, cytokine and ECM production. TGF- β signaling in epithelial cells blocks transcription of *c-myc*, which is important for growth inhibition (Moses et al., 1991). The tumor cells circumvent the inhibitory action for example through inactivation of core components, such as mutations of the type II TGF- β receptor, T β RII, or SMAD4, of the signaling pathway regulating the specific trait turning it into a tumor promoting property. However, when these are mutated in mice it is not sufficient for tumor formation and another stimulus such as tissue injury for example, which change the environment inducing proliferative cues, is needed (Massague, 2008; Tian and Schiemann, 2009). This can be exemplified by a study where the T β RII was mutated in mammary epithelia. The mere deletion induced hyperplasia and increased apoptosis but when combined with the MMTV-PyMT mouse model tumors were detected earlier and metastasis was enhanced (Forrester et al., 2005). Endothelial cells may respond with proliferation, migration and morphogenesis upon TGF- β stimuli or the opposite but this will be discussed in more details later (Goumans et al., 2003a). Fibroblasts are also important targets for TGF- β signaling in tumors where signaling affects ECM production, proliferation and cytokine secretion. As opposed to T β RII inactivation in epithelial cells not being enough for malignant transformation, T β RII deletion in FSP-1 expressing fibroblasts induced squamous cell carcinoma in the forestomach through upregulation of HGF (Bhowmick et al., 2004a). TGF- β is also a major player in EMT and EndMT and has effects on the immune system as it is a key component in the induction of immune tolerance (Massague, 2008; Tian and Schiemann, 2009). The implications of TGF- β signaling in cells other than endothelial cells will not be discussed further. In summary, the TGF- β signaling and effects are highly complex and the outcome of manipulating the system may yield unexpected and difficult to interpret results. With the use of the new and complex mouse models we are starting to understand more about this intriguing system and the deciphering will continue.

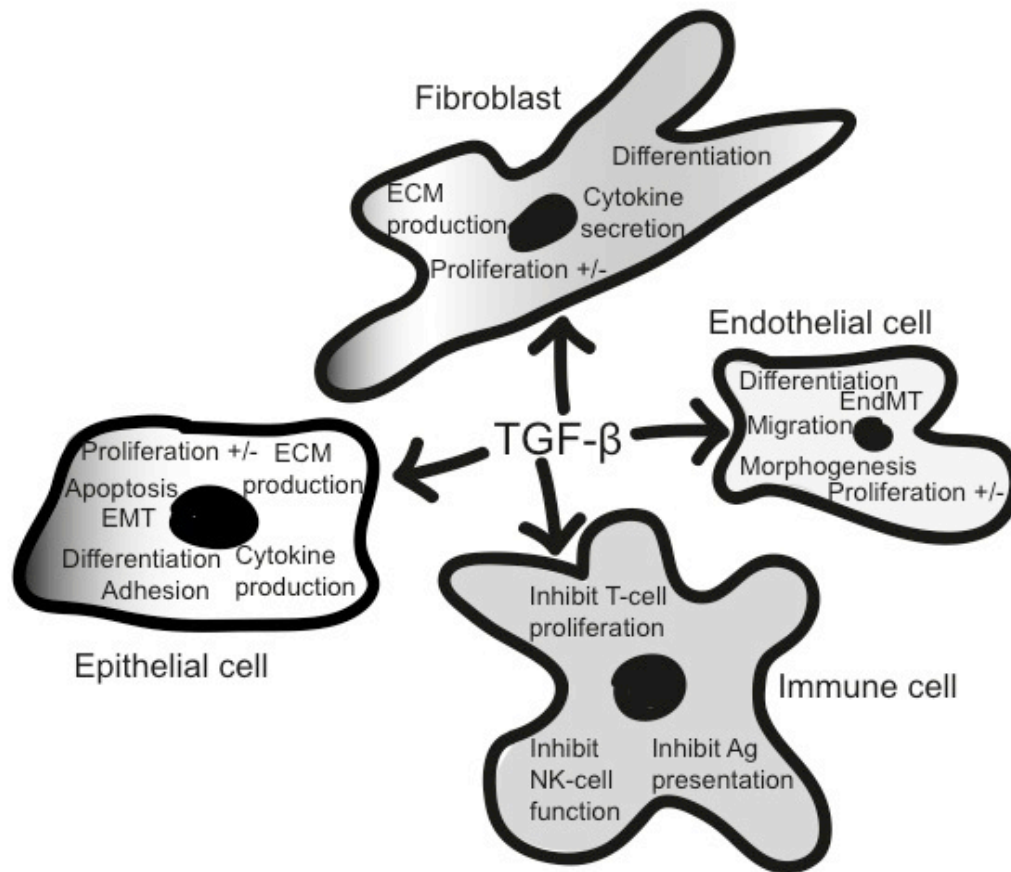


Figure. 4. TGF-β effects on different cell types.

TGF-β exerts a number of diverse effects in a context dependent manner on many cell types, reflecting its dual role in tumor biology.

1.3.2.1 Endoglin

Endoglin (also CD105) is a type III TGF-β co-receptor. It is highly expressed in endothelial cells during active angiogenesis whereas it is expressed at low or non-detectable levels in non-active endothelial cells (Burrows et al., 1995; Fonsatti et al., 2000; Miller et al., 1999). Endoglin is considered as an endothelial cell marker but is expressed on some other cell types, mostly under certain specific conditions such as on monocytes during monocyte-macrophage transition, syncytiotrophoblasts in the placenta at term, a subset of neural crest stem cells during development, adult bone marrow hematopoietic stem cells and mesenchymal stem cells (Schieker et al., 2007; ten Dijke et al., 2008). The receptor has a large extracellular domain and a short cytoplasmic domain. There are two isoforms, long- and short-endoglin with the length of the cytoplasmic tail deviating. The long form is the preferentially expressed variant, particularly in the endothelium (Perez-Gomez et al., 2010). Endoglin is also present in a soluble form and MMP-14 was recently shown to be responsible for producing soluble endoglin from the membrane bound form (Hawinkels et al., 2010). Out of the large TGF-β family ligands, endoglin binds to TGF-β1, TGF-β3, activin-A, BMP-2, BMP-7, BMP-9 and BMP-10. However, BMP-9 and BMP10 are the only ligands that membrane bound endoglin can bind to without the presence of the TGF-β

type I and type II receptors (Barbara et al., 1999; Bellon et al., 1993; Castonguay et al., 2011; Cheifetz et al., 1992; Letamendia et al., 1998; Scharpfenecker et al., 2007).

When endoglin expression is ablated in mice challenged with tumors, these tumors have displayed a reduced growth rate as a result of lower vascular density (Duwel et al., 2007). Tumor models where endoglin function is inhibited using antibodies or vaccine strategies have displayed similar phenotypes with reduced tumor growth and lower vascular density, sometimes in combination with diminished metastasis (Seon et al., 1997; Takahashi et al., 2001a; Tan et al., 2004; Uneda et al., 2009). Endoglin is commonly up-regulated in the tumor vessels in several different tumor types and has been shown to have prognostic significance (Burrows et al., 1995; Charpin et al., 2004; Kumar et al., 1999; Martone et al., 2005; Wikstrom et al., 2002). Soluble endoglin seems to act as an inhibitor of endoglin effects (Castonguay et al., 2011; Hawinkels et al., 2010) (Hawinkels 2010, Castonguay et al, JBC 2011) and high levels of circulating endoglin have been reported to correlate to occurrence of metastasis in breast and colon carcinoma (Takahashi et al., 2001b). Endoglin inhibitors are under investigation and the human chimeric antibody TRC105 (Matsuno et al., 1999) has entered clinical trials.

1.3.2.2 ALK1

ALK1 is a TGF- β type I receptor with expression restricted to the endothelium. The ligands of ALK1 were unknown for quite some time but to date BMP9, BMP10, TGF- β 1 and TGF- β 3 have been shown to interact with ALK1 under some conditions (David et al., 2007; Goumans et al., 2003b; Goumans et al., 2002; Suzuki et al., 2010). It is however debated which is the true ALK1 ligand (David et al., 2009). Activation of ALK1 induces phosphorylation of receptor-regulated SMAD1/5/8 as opposed to receptor-regulated SMAD2/3 which is induced by ALK5 signaling (van Meeteren and Ten Dijke, 2011).

It is to date not fully elucidated how ALK1 modulates angiogenesis. There are many contradictory reports and several levels of complexity. Despite this, several means of inhibiting ALK1 signaling, both through kinase inhibitors and soluble receptor analogues, are currently under investigation (Cunha and Pietras, 2011).

1.3.2.3 TGF- β family signaling in the endothelium

TGF- β signaling is highly dependent on context and cellular differentiation stage. The complex results of TGF- β signaling in the vessels have not been thoroughly investigated and it is also common with contradictory results. Part of the explanation to the many contradictory results obtained is that many studies have been performed *in vitro* with emphasis on one ligand and/or receptor family member not taking into account the complexity in the *in vivo* situation where multiple ligands and multiple receptors may be present in a number of different combinations. However, the *in vitro* studies are also very important in order to study the complex effects of TGF- β signaling but should be designed with great attention on details, such as ligand availability of the specific site *in vivo*. We are still lacking many very important pieces of information to explain the TGF- β family's intricate, diverse and contradictory effects in angiogenesis.

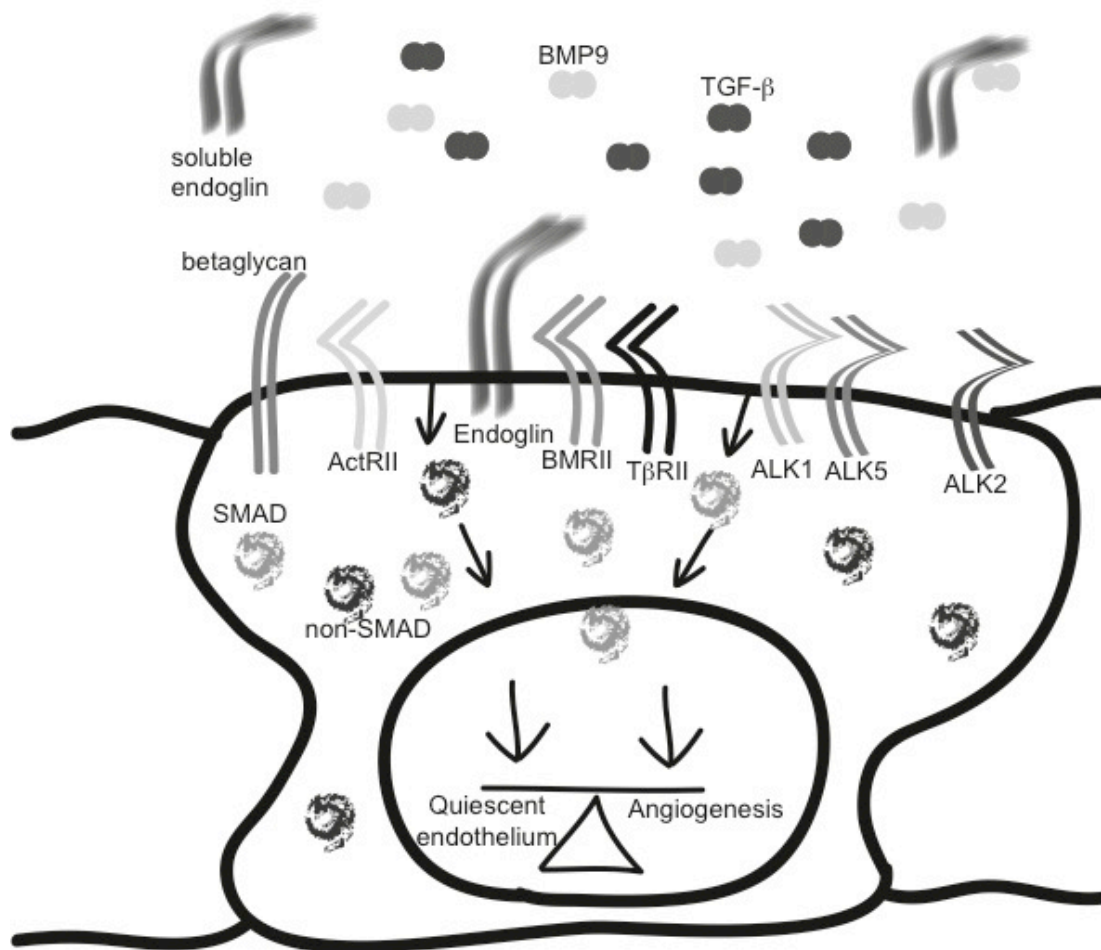


Figure 5. Schematic illustration of the TGF- β signalling components in the endothelium.

Endothelial cells have been shown to express a number of different TGF- β receptors. Expression of three different type I receptors, ALK1, ALK2 and ALK5, three type II receptors, TβRII, BMPRII and ActRII in addition to two type III receptors, endoglin and betaglycan have been reported.

Endothelial cells express two different TGF- β type I receptors, the ubiquitously expressed ALK5 and the endothelial restricted ALK1. Endothelial cells may also express ALK2 and the co-receptors endoglin and betaglycan (David et al., 2009; David et al., 2007; van Meeteren and Ten Dijke, 2011). Deletion experiments in mice have shown a high degree of similarity between phenotypes when ALK1, ALK5, Endoglin and TβRII are knocked out. They are all embryonically lethal around mid gestation due to vascular defects (ten Dijke and Arthur, 2007). This suggests that they all have important functions that are non-redundant in the developing embryo, which also may translate into important functions during tumor angiogenesis. Interestingly, functional mutations resulting in a heterozygote phenotype of endoglin or ALK1 are found in the human disease hereditary haemorrhagic telangiectasia (HHT). The phenotype is slightly different between the variants but is characterized by arteriovenous malformations and telangiectasia induced bleedings in the gut and nose (Shovlin, 2010).

TGF- β 1 binds both to the ALK1 and ALK5 receptor in the endothelial cell. It has been proposed that these two receptors have opposite effects on angiogenesis directing signaling either towards pro-angiogenesis with migration and proliferation or a quiescent state, respectively. To dissect this constitutively active ALK1 or ALK5 receptors were expressed in endothelial cells and subject to microarray analysis revealed some specific target genes for each receptor, plasminogen activator inhibitor type 1 (PAI-1) and fibronectin for ALK5 and inhibitor of DNA binding (ID)1 and interleukin 1 receptor like 1 (IL1RL1) for ALK1 (Goumans et al., 2002). Interestingly, PAI-1 is an inhibitor of angiogenesis *in vivo* (Stefansson et al., 2001) and ID1 has been shown to enhance migration, invasion and proliferation in endothelial cells while IL1RL1 is suggested to be involved in cell growth (Goumans et al., 2003a; Goumans et al., 2002; Lin et al., 2000). They suggest that ALK1 has growth stimulatory effects on endothelial cells. However, another study showed that the constitutively active ALK1 inhibited cell cycle progression suggested that ALK1 is important for the maturation stage of vessel formation (Lamouille et al., 2002). The two studies also reported conflicting results regarding *c-myc* expression. The constitutively active ALK5 rendered endothelial cells with reduced migration and proliferation, which they implicate in vessel maturation (Goumans et al., 2002). Explanations for the discrepancy of the results may be the use of different cell lines and also culturing conditions. The role of endoglin in this signaling complex has been described as modulating the signaling in favour of ALK1 and inhibiting ALK5 (Goumans et al., 2002). Knockdown of endoglin in endothelial cells caused suppression of growth and migration indicating that endoglin presence is important for angiogenesis (Li et al., 2000a). Adding strength to the suggested balance where ALK1 and endoglin enhance while ALK5 suppresses angiogenesis is the similarities seen between knockdown of endoglin and ALK1 *in vivo*. The knockouts display very similar phenotypes and the corresponding human diseases, HHT1 and HHT2 respectively, also display overlapping symptoms. Efficient signaling by ALK1 has also been shown to require the presence of ALK5 in cultured endothelial cells. Endothelial cells lacking ALK5 failed to respond to TGF- β stimulation even in the presence of ALK1 or overexpressed ALK1 (Goumans et al., 2003b). These results were not supported by another study where endothelial conditional knockout mice of ALK1, ALK5 and T β R II were compared. The ALK1 deletion exhibited vascular malformations while the conditional knockouts did not indicating that ALK1 is not dependent on ALK5 presence (Park et al., 2008).

2 AIMS

The tumor's stroma, composed of many different cell types, contain many, yet unidentified, signaling pathways and processes that we conceive are promising new druggable targets. The tumor micro- and macroenvironment are highly plastic compartments with the ability to adapt to the insults of treatment. By simultaneously targeting multiple signaling pathways and cell types, we believe that cancer can be treated more efficiently than today with perhaps less side effects.

To achieve this, we have to improve our current understanding of the complex and intricate molecular interactions that governs paracrine signaling in the tumor microenvironment. Specifically, molecular interactions and paracrine signaling by CAFs and endothelial cells will be explored *in vivo*. We aim to investigate the dependence of these cell types on signaling through members of the PDGF family and the TGF- β family respectively.

Specific aims:

- To investigate the effect of paracrine PDGF-CC signaling on the tumor microenvironment (Paper I).
- To investigate the significance of PDGF-CC expression in breast cancer and explore the therapeutic opportunities of monoclonal PDGF-C antibodies (Paper II).
- To elucidate the function of TGF- β signaling in the tumor microenvironment, focusing on the effects of ablated TGF- β signaling via the endothelial receptors endoglin (Paper III) and ALK1 (Paper IV).

3 RESULTS AND DISCUSSION

3.1 PAPER I: PARACRINE SIGNALING BY PLATELET-DERIVED GROWTH FACTOR-CC PROMOTES TUMOR GROWTH BY RECRUITMENT OF CANCER-ASSOCIATED FIBROBLASTS.

Several studies have implicated PDGFs as prominent factors creating a growth promoting tumor stroma. Most studies have focused on the classical PDGFs and especially PDGF-BB. Tumors expressing PDGF-BB display a reduced latency time and present with increased number of vessels and fibrosis (Forsberg et al., 1993). In addition, paracrine PDGF-BB has been shown to create a growth permissive stroma for keratinocytes (Skobe and Fusenig, 1998). Later, stimulation of CAFs producing VEGF initially and later HGF was identified as the driving force for the creation of the permissive stroma (Lederle et al., 2006). PDGF-AA has been implicated in CAF recruitment to tumors as down regulation of *pdgf-a* in tumor cells reduced the desmoplastic response (Shao et al., 2000). In addition, another tumor model with abundant stroma was shown to express high levels of PDGF-AA. Inhibition of PDGFR- α reduced the tumor growth rate. Elevated *pdgf-c* gene expression was also noted but the relative contribution of PDGF-AA and PDGF-CC was not addressed (Tejada et al., 2006). However, there is no information regarding the mechanism whereby this occurs, where the cells are recruited from and the molecular functional consequences in the tumors. Thus, the classical PDGFs are important for the stimulation of the stromal compartment, creating a tumor permissive microenvironment.

Less is known about the importance of paracrine signaling in tumors by the novel PDGFs. PDGF-CC has been shown to induce fibrosis in tissues such as liver, heart and kidney (Campbell et al., 2005; Eitner et al., 2008; Ponten et al., 2003). The expression of PDGF-CC is increased in several different skin cancers compared to the normal situation. Therefore, we wanted to study the effect of paracrine PDGF-CC signaling in a syngeneic tumor model.

We started by confirming PDGF-CC expression and expression pattern of the receptor, PDGFR- α , in normal skin and malignant melanoma. Tumor cells in melanoma expressed PDGF-CC whereas PDGFR- α was present in cells of fibrotic streaks with fibroblast morphology. In normal skin, PDGF-CC expression was restricted to hair follicles and PDGFR- α expression was weak.

We decided to express PDGF-CC in B16 melanoma cells, which are syngeneic to C57Bl/6 mice. These cells did not appear to be affected by the expression of PDGF-CC *in vitro*. However, when injected subcutaneously, they grew significantly faster. The tumors expressing PDGF-CC exhibited increased proliferation as shown by BrdU incorporation in combination with augmented vessel density. The apoptotic rate was reduced and there was no difference in pericyte coverage of vessels when assessed with the pericyte marker NG2.

We noted an increased thickness of the fibrous capsule surrounding B16/PDGFC tumors. This capsule contained PDGFR- α positive cells. To investigate this in more detail we established B16 PDGF-CC expressing and control tumors in PDGFR- α /GFP mice where one allele of PDGFR- α is substituted by GFP. This allowed us to identify a GFP positive population of cells infiltrating the tumor edge in PDGF-CC expressing tumors. The GFP positive cells were identified as fibroblasts by their expression of the fibroblastic marker FSP-1, which was present both at the tumor edge and in the tumor parenchyma. The abundant FSP-1 expression in the B16 tumors was dependent on activation of PDGFR- α as B16 tumors formed from cells expressing PDGF-BB was devoid of FSP-1 infiltrating cells.

To identify the proteins that were responsible for the increased tumor growth rate in PDGF-CC expressing tumors we used antibody arrays allowing simultaneous assessment of 96 different proteins. OPN was highly expressed in the PDGF-CC tumors and in addition, FGF-2 expression was also increased. Cells of the fibrotic capsule and single infiltrating cells in the tumors were found to express OPN in immunohistochemical stainings.

Three different subsets of CAFs were identified using FSP-1 staining in the tumors from PDGFR- α /GFP mice. At the tumor edge, cells expressing high PDGFR- α were present together with a second subset of PDGFR- α low expressing cells co-expressing FSP-1. In the center of the tumor, the third subset only expressing FSP-1 was identified. All these populations expressed α -SMA to various degrees. The combination of markers expressed identified these three populations of cells as CAFs. Co-stainings with OPN revealed that the predominant cells producing it were CAFs of the PDGFR- α low/FSP-1 subclass. However, some FSP-1 expressing cells also expressed OPN.

The functional dependence of the B16 tumors on OPN was investigated using co-injections of B16 cells and mouse embryonic fibroblasts (MEFs) derived from wild type or OPN deficient mice. Co-injection of tumor cells with wild type MEFs reduced the tumor latency time and increased the tumor growth rate while OPN deficient MEF tumors displayed latency time and tumor growth rate comparable to injection of tumor cells alone. Thus, identifying OPN as a functional important protein for melanoma growth.

From these studies we concluded that PDGF-CC expression resulted in an increased recruitment of CAFs to the tumor. These CAFs expressed OPN, which induced an increased tumor growth rate. OPN is a ubiquitously expressed secreted protein. It is commonly expressed in tumors and associated with poor prognosis (Anborgh et al., 2010). CAFs have in many different systems been implicated in angiogenesis, be it directly or indirectly, as they have been shown to secrete factors such as VEGF, CXCL14 and CXCL12/SDF-1 (Augsten et al., 2009; Fukumura et al., 1998; Orimo et al., 2005). OPN has been shown to induce angiogenesis and the effect was potentiated when FGF-2 was present (Leali et al., 2003). Interestingly these are the two differentially expressed proteins in our system when PDGF-CC recruits fibroblasts.

The angiogenic factor FGF-2 has been found to be important for tumor growth in a cervix cancer mouse model. FGF-2 expression was ablated when PDGF signaling was blocked using tyrosine kinase inhibitors (Pietras et al., 2008). In line with these results we also found that FGF-2 expression by CAFs increased in PDGF-CC expressing tumors. OPN has been shown to be important for recruiting bone marrow derived cells (BMDC), which induced a growth promoting stroma through activation of CAFs. These CAFs were not recruited from the bone marrow (Elkabets et al., 2011; McAllister et al., 2008). OPN is also found on the stromal gene list produced by Finak and colleagues that is associated with poor outcome in breast cancer (Finak et al., 2008).

We speculate that the three different CAF populations that we identified in the tumors may be the result of education of a cell type that expresses PDGFR- α . This could be resident fibroblast but intriguingly, MSC also express PDGFR- α (Ball et al., 2007). We hypothesise that PDGFR- α expressing cells are educated by the tumor microenvironment to evolve into CAFs expressing the effector proteins we identified in this study, OPN and FGF-2. Another option is that OPN itself is responsible for recruiting BMDC that in turn are responsible for the observed phenotype through an indirect mechanism.

Another study has shown that CAFs expressing FSP-1 are able to induce metastasis through the effector molecules, VEGF and tenascin-C (O'Connell et al., 2011). It would be interesting to investigate whether metastasis is increased in our system as OPN has been implicated in induction of a growth promoting stroma at distant sites (McAllister et al., 2008). OPN expression has also been shown to be up-regulated in metastases of melanoma (Jaeger et al., 2007).

We suggest that tumor growth will be reduced if PDGF-CC is targeted which presumably would reduce the content of OPN and FGF-2 in PDGF-CC expressing melanomas. PDGF-CC signaling can be targeted through use of tyrosine kinase inhibitors such as imatinib, sunitinib and sorafenib, which already are used in the clinic (Board and Jayson, 2005). Alternatively, OPN and FGF-2 could be targeted. These effects may not be restricted to melanoma alone, but would presumably display therapeutic potential in other tumors where PDGF-CC is present and involved in recruiting an activated stroma.

3.2 PAPER II: HIGH EPITHELIAL PDGF-CC EXPRESSION PREDICTS CLINICAL OUTCOME IN BREAST CANCER.

We have previously identified PDGF-CC as an important growth factor for recruitment CAFs to melanoma in a mouse model. PDGF-CC was also found to be up-regulated in several human skin tumors of different types and the corresponding receptor PDGFR- α was found in the fibrotic streaks of malignant melanomas (Paper I). During the initiation of this project PDGF-CC expression was assessed in ten different tumor types. Breast tumors displayed highly increased PDGF-CC expression compared to normal breast tissue.

Apart from general clinical characteristics such as tumor grade, size and lymph node status the expression of hormone and growth factor receptors; estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) hold prognostic information and guide the use of targeted therapies in breast cancer (Gradishar, 2005). We set out to investigate whether PDGF-CC expression in breast cancer could be used as a prognostic marker and possibly pose as a target for therapy.

First, a monoclonal PDGF-C antibody, clone 1, was developed. Clone 1 recognized human PDGF-CC, both latent and activated form, on western blot. It did not show immunoreactivity towards PDGF-DD. Clone 1 blocked ligand-induced phosphorylation of PDGFR- α *in vitro* and inhibited PDGF-CC stimulated cell growth.

PDGF-CC expression was then characterized using clone 1 on tissue microarrays of a large (890 patients) patient cohort. The stromal and epithelial expression were scored separately as both stromal and epithelial cells expressed PDGF-CC. PDGF-CC expression increased during tumor progression and expression of PDGFR- α was confirmed in the stromal compartment in a selected subset of tumors. Epithelial PDGF-CC expression was shown to be positively correlated with tumor grade, cytokeratin 5/6, HER2 and proliferation assessed by Ki-67 in Spearman rank analyses. Negative correlations were found for ER, PR and menopausal status. Epithelial PDGF-CC expression displayed independent prognostic value for overall survival. Interestingly, PDGF-CC expression correlated with marker characteristics that have been assigned to basal-like breast cancer. As PDGF-CC expression correlated positively with epidermal growth factor receptor and cytokeratin5/6 and negatively with ER, PR and tumor grade this suggest that PDGF-CC may be a marker of basal like breast cancer. Basal-like breast cancer patients generally have an unfavourable prognosis with poor treatment response to traditional dosing schedules (Gluz et al., 2008). This also correlates with the poor prognosis of patients expressing epithelial PDGF-CC.

We investigated the effects of PDGF-CC in an orthotropic breast tumor model. The human MDA-MB-231 cell line was shown to have high *pdgf-c* expression and is known to have basal characteristics. Cells were injected into the 4th mammary fat pad and allowed to form palpable tumors. The mice were treated twice weekly with intraperitoneal injections of clone 1 or isotype matched control antibody. Inhibiting epithelial PDGF-CC reduced tumor growth rate and at the endpoint, tumor size was 30% less in the treated cohort. These tumors also had a reduced vessel number, as assessed by podocalyxin staining.

We conclude that high epithelial PDGF-CC expression in breast cancer is associated with a shorter survival. Epithelial PDGF-CC was found to be an independent prognostic marker. Inhibiting PDGF-CC signaling in our mouse model reduced the tumor growth rate using a monoclonal antibody as a mono-therapy. We suggest that PDGF-CC is a marker of basal-like breast cancer and that inhibiting PDGF-CC signaling in basal-like breast cancer may be a novel therapy option in a form of breast cancer currently lacking targeted therapy. In addition, epithelial PDGF-CC expression represents an independent prognostic factor of survival.

Further investigations to dissect the role of PDGF-CC expression in breast cancer are warranted. The molecular mechanism needs to be investigated. In light of our previous results with OPN expression increasing when PDGF-CC is expressed it would be interesting to investigate if OPN is involved in this system. High OPN was found to be a marker of poor overall survival in a meta-analysis in all tumors combined and also in breast, lung and prostate cancer (Weber et al., 2010). Investigating the metastatic pattern in light of PDGF-CC expression, especially if OPN is high in the PDGF-CC expressing tumors, would also be interesting. OPN has been shown to promote CCL5 expression in mesenchymal stromal cells and that the abundance of these cells is increased at the site of metastasis in their mouse model by an OPN dependent mechanism (Mi et al., 2011). Finally, OPN was found among the up-regulated stromal genes predicting poor outcome in breast cancer (Finak et al., 2008).

We are currently studying the effect of genetic ablation of PDGF-CC in a genetically modified mouse model of breast carcinoma. In short, PDGF-CC deficient mice were crossed with the MMTV-PyMT breast tumor model. Initial characterization of this model revealed a delayed onset of tumor growth and over 50 % reduction in tumor size in mice heterozygote for *pdgf-c*.

3.3 PAPER III: ADAPTATION TO IMPAIRED TUMOR ANGIOGENESIS IN ENDOGLIN-DEFICIENT MICE IS PARALLELED BY A WEAKENED ENDOTHELIAL BARRIER TO METASTATIC DISSEMINATION.

The introduction of antiangiogenic therapies into the clinic did not meet the high expectations on increased overall survival predicted from preclinical studies. The common theme of all introduced angiogenic-targeting therapies are inhibition of VEGF signaling. However, recent preclinical trials have described several means of resistance to the VEGF inhibition (Bergers and Hanahan, 2008; Casanovas et al., 2005; Ebos et al., 2009a; Ebos et al., 2009b; Paez-Ribes et al., 2009).

Are these resistance mechanisms exclusive to anti-VEGF therapy? Could similar mechanisms be identified when manipulating other signaling pathways involved in angiogenesis in tumors? High expression of endoglin in tumor endothelial cells has been reported and high endoglin expression correlates with poor survival (Charpin et al., 2004; Dales et al., 2004; Kumar et al., 1999; Martone et al., 2005; Takahashi et al., 2001b; Wikstrom et al., 2002). Consequently, we wanted to investigate the role of the TGF- β co-receptor endoglin in tumor angiogenesis.

We started our investigations by characterizing the expression of endoglin in the prototypical genetically engineered mouse RIP1-Tag2 model that develop pancreatic neuroendocrine tumors in a multistep process in a predictable manner. Endoglin was shown to be expressed by the tumor vessels in this model and exhibited a peak in expression in the angiogenic lesions. The RIP1-Tag2 mouse was crossed with endoglin haploinsufficient mice. Initial characterization of the tumor development in the deficient mice revealed a reduction in number of angiogenic islets and number of tumors but the tumor volume was similar. This indicates that even though fewer lesions progressed to the angiogenic and tumor stage, the ones that did evade the angiogenic insult displayed a growth aggressive phenotype. There was no difference in vessel density assessed by podocalyxin nor perfused vessels when using lectin.

To confirm the phenotype we used a syngeneic breast tumor cell line, EO771, that we injected orthotopically in endoglin haploinsufficient mice and controls. Endoglin deficiency did not affect the growth pattern of these tumors indicating that they were intrinsically resistant. Similar to the RIP1-Tag2 model, there was no change in vessel density. In addition, we used an inducible endothelial cell specific endoglin knockout mouse model injected subcutaneously with LLC cells. Initial growth impairment was recorded 6 days after injections but the tumors circumvented this and mice presented with similar tumor volumes at the end point. The vessel density showed a similar pattern, reduced in endoglin knockouts at 6 days but equal at the end point.

We recorded an increase in micro- or macrometastases to the respective target organ in all three mice models. Thus the resistant tumors displayed an increased metastatic capacity.

Next, we investigated whether concurrent treatment with VEGF inhibitors of endoglin haploinsufficient mice bearing tumors would affect tumor growth, as the observed phenotype resembled the phenotype of VEGF inhibition. We selected a long treatment

schedule that would allow the tumors to become resistant to the anti-VEGF therapy. Interestingly, the combination of genetic endoglin deficiency and anti-VEGF treatment did not seem to induce resistance and mice presented with smaller tumors (RIP1-Tag2) or reduced tumor growth rate (EO771). This also translated into a decreased number of detected micrometastases to a similar level as seen without angiogenic insult.

There are several possible explanations to the increased number of micrometastases. It could be the tumor cells that acquire increased ability to metastasize through up-regulation of e.g. migratory or invasive genes or changes in the endothelial cells facilitating transmigration of tumor cells. We did not detect any differences in the tumor cells. We therefore turned our interest to the endothelial barrier function. Knockdown of endoglin in the endothelial cell line bEND3.1 resulted in an increased tumor cell transmigration of cells from RIP1-Tag2 tumors (β TC-3) and EO771 cells over the endothelial cell barrier in a transwell assay. We detected an increase in transcripts from circulating tumor cells in blood from endoglin haploinsufficient RIP1-Tag2 mice suggesting enhanced transmigration of tumor cells also *in vivo*. We could not detect any difference in number of β TC-3 cells extravasating in the liver of endoglin deficient mice after tail vein injections. This implies that a change in the endothelial barrier of the tumors in RIP1-Tag2 mice is responsible for the increased metastatic potential when endoglin is ablated. However, when LLC cells injected in the tail vein in endothelial endoglin knockout mice an increase in number of lesions in the lung was detected. These, at first view contradictory results, may be explained by the distribution of endoglin expression in the respective organ, liver has very little endoglin expression while the lungs display high expression.

We continued the investigations by looking at the vessels in the RIP1-Tag2 tumor model in closer detail. The expression pattern of the endothelial junctional protein CD31 was changed. The CD31 strongly positive vessels were reduced in endoglin deficient mice and in cases where CD31 appeared strong, the staining pattern was more diffuse not closely associated with the podocalyxin co-staining. To investigate if this reduction of an endothelial junctional marker was a sign of the endothelial cells losing their identity through EndMT we stained tumors for α -SMA, a mesenchymal marker. α -SMA staining was strongly induced in some areas in endoglin deficient mice surrounding the vessels and also sometimes overlapping with the vessel markers podocalyxin and CD31. Increased abundance of α -SMA in association with or overlapping with vessel marker was also detected in EO771 tumors from endoglin deficient mice.

We next isolated endothelial cells from RIP1-Tag2 tumors of each genotype and found the transcription factor twist up-regulated in endoglin deficient endothelium on the mRNA level. Twist is a key activator of EndMT. EndMT is characterized by loss of cell-cell junctions and acquisition of invasive and migratory properties. Concomitantly, endothelial markers such as CD31 is lost and mesenchymal markers like FSP-1 and α -SMA are up-regulated (Potenta et al., 2008). Most knowledge about EndMT have been collected from developmental studies but one study show that a substantial proportion of CAFs was derived through EndMT in the B16 mouse melanoma model (Zeisberg et al., 2007). TGF- β signaling is a known inducer of EndMT (Potenta et al., 2008). We continued to investigate the EndMT phenotype of endothelial cells *in vitro* in the

context of endoglin signaling. Knock down of endoglin in bEND3.1 cells stimulated with TGF- β displayed an increase in the mesenchymal marker α -SMA and concurrent decrease of endothelial marker CD31 both on western blot and in immunostainings. Using the transwell assay system we showed that TGF- β stimulation of a MS1 endothelial cell layer increased the transmigration of β TC-3 cells. In addition, knock down of CD31 also increased the transmigration of β TC-3 and EO771 cells. The increased transmigration of tumor cells suggest that induction of EndMT characteristics in endothelial cells induced changes that weakened the endothelial cell barrier.

In conclusion, genetic ablation of endoglin expression only inhibited tumor growth transiently. The lesions adapted to the angiogenic insult and developed resistance. The resistance phenotype was associated with increased metastatic seeding to distant organs. Vessels in the tumor did go through EndMT rendering them more permissive to tumor cell transmigration enabling the metastatic phenotype. In addition, combining two angiogenic insults, VEGF inhibition and genetic ablation of endoglin maintain the tumors sensitive to anti-VEGF therapy resulted in reduced tumor volume and decreased seeding of distant metastases. Our results indicate that combining two different antiangiogenic insults, at least, prolongs time until the tumors show signs of resistance with regard to tumor growth and metastatic capacity. This suggests that a combination of antiangiogenic therapies should be investigated in the human setting to avoid the induction of resistant tumors and possibly increase the overall survival. We also identified EndMT induced by genetic ablation of endoglin singling having important function in the tumor vessel integrity and hence the metastatic process in tumors.

The knowledge about EndMT is still rather limited and especially in tumor biology. However, TGF- β signaling has been shown to be important in EndMT (Potenta et al., 2008). It was recently shown that TGF- β mediated ALK5 dependent signaling induced by shear stress in endothelial cells that were sensitized by the lack of cilia induced EndTM (Egorova et al., 2011). This may suggest that the EndMT we detect are a result of increased ALK5 signaling due to the lack of endoglin modulation of signaling (ten Dijke et al., 2008).

EndMT may be the explanation to the noted increase in α -SMA expressing cells in the vicinity of vessels in a subset of tumors after anti-VEGF therapy inducing resistance (Franco et al., 2011a; Helfrich et al., 2010). This phenomenon has also been described in RIP1-Tag2 mice deficient for neural cell adhesion molecule (NCAM) (Xian et al., 2006), implicating reduction of NCAM in EndMT.

Aberrant endoglin has previously been correlated with increased metastasis in human studies (Takahashi et al., 2001b). In these cases soluble endoglin have been elevated which is suggested to act as an inhibitor of cell surface bound endoglin modulation of signaling (Hawinkels et al., 2010). HHT1 patients are deficient in endoglin and our endoglin heterozygot mice mimic their disease (Shovlin, 2010). Our result suggest that this patient group, in case of tumor development, would have an increased risk of metastatic disease and would benefit from anti-VEGF therapy. We propose that further studies are warranted regarding the effect of combining inhibition of different antiangiogenic signaling pathways simultaneously in order to induce a sustainable

antiangiogenic effect without the development of resistance resulting in an increase in overall survival.

3.4 PAPER IV: GENETIC AND PHARMACOLOGICAL TARGETING OF ACTIVIN RECEPTOR-LIKE KINASE 1 IMPAIRS TUMOR GROWTH AND ANGIOGENESIS.

TGF- β and BMP-9 share a common TGF- β type I receptor, ALK1. Contradictory reports regarding the inhibitory or stimulating effect TGF- β and BMP-9 have on angiogenesis prompted us to investigate the effect of modulating this system *in vivo*, with the aim of investigating this system as a potential pathway to interfere with tumor angiogenesis.

We started by characterizing the expression of ligands and receptors of the tumors of RIP1-Tag2 mice. The expression, assessed by quantitative RT-PCR, of TGF- β and BMP-9 increased during the tumorigenic process and ALK1 expression was increased in the angiogenic lesions. Histological examination revealed that both ligands were expressed predominantly by the tumor cells. ALK1 expression was restricted to the endothelial cells in vessels and was not present in pericytes.

Mice lacking one copy of the *alk1* gene were bred with RIP1-Tag2 mice to investigate the effect of genetically ablated ALK1 signaling during tumorigenesis. The number of angiogenic lesions was reduced and this translated into fewer tumors and lower additive tumor volume. Thus, the angiogenic switch in the ALK1 deficient mice was retarded translating into slow down of the tumorigenic process. When assessing the tumor vascular density it was reduced and also the density of perfused vessels was lower in the ALK1 deficient mice.

We assessed the option of using ALK1 inhibition as a therapeutic target using an ALK1-Fc fusion protein, RAP-041. *In vitro* studies revealed that RAP-041 was a potent inhibitor of BMP-9. The fusion protein also affected VEGF and FGF-2 induced angiogenesis in the matrigel plug assay reducing the ingrowth of vessels. When RIP1-Tag2 mice were treated with RAP-041, tumor volume was reduced in a dose dependent manner. A two week long treatment schedule, started both at early (at 10 weeks) and late (at 12 weeks) stages of disease, showed that the inhibitor blocked tumor growth. The treated animals presented with a tumor burden comparable to a control cohort sacrificed at the initiation of treatment. Histological characterization of treated mice or controls, regardless of tumor stage, revealed a reduced vessel density after the treatment as shown by CD31 staining and also the perfused vessel density was lower. Hence, RAP-041 was able to inhibit tumor angiogenesis in our mouse model resulting in increased apoptosis that augmented tumor growth.

The molecular mechanism was investigated *in vitro*, *ex vivo* and *in vivo*. The effect of either ligand alone or in combination was studied in three different cell lines. The combination of TGF- β and BMP-9 stimulated endothelial cell proliferation when VEGF was present but either ligand alone reduced the proliferation. Using the matrigel plug assay with baseline presence of both VEGF and FGF-2 and variable presence of TGF- β or BMP-9 or the combination *in vivo* confirmed the phenotype of the *in vitro* experiments. The combination of ligands improved the ingrowth of vessels. This effect was blocked by RAP-041. To further strengthen the observation that the combination induced angiogenesis while either ligand alone had the opposite effect an *ex vivo*

sprouting angiogenesis assay was designed. In short, angiogenic islets from RIP1-Tag2 mice were isolated and seeded onto matrigel plugs containing endothelial cells, the ligands of interest were added to the culturing media. It is known that angiogenesis in the RIP1-Tag2 tumors are dependent on VEGF and the angiogenic islets contain VEGF (Inoue et al., 2002). Again the combination induced a positive response with endothelial cell sprouting and migration while either ligand alone or addition of RAP-041 negated the effect. Thus, the combination of TGF- β and BMP-9 has a synergistic effect enhancing angiogenesis prompted by VEGF and FGF-2.

We also analysed the expression pattern of genes with pro-angiogenic effects downstream of ALK1 (ID1 and ID3) and ALK5 (PAI-1) signaling by quantitative RT-PCR. In tumors from RIP1-Tag2 mice that were either ALK1 heterozygote or treated with RAP-041, expression levels of all targets genes were down-regulated. In line with this, endothelial cells in culture up-regulated expression of PAI-1 in response to the combination of TGF- β and BMP-9, both on RNA and protein level. Interestingly, the synergistic effect of both ligands required both ALK1 and ALK5 receptors as specific inhibition of each receptor with the combination of both ligands reduced the expression of the target gene PAI-1. Assessing activation of upstream signaling pathways suggested that SMAD2 was involved. In conclusion, target genes downstream of both ALK1 and ALK5 were affected by reduction of ALK1 signaling through genetic or pharmacological targeting.

Genetic ablation or pharmacological targeting of ALK1 demonstrates that tumor angiogenesis is reduced and tumor growth halted. Hence, targeting of ALK1 represents a valid therapeutic target of antiangiogenic therapy. We believe that some of the discrepancies reported in earlier studies of TGF- β family effects on angiogenesis might be explained by the presence or absence of unexpected or not studied receptors and/or ligands. Most likely, a correct delicate balance of both receptors and ligands affect the outcome of TGF- β family signaling in angiogenesis.

The humanized form of RAP-041, ACE-041, and a monoclonal antibody are initiating clinical trials as antiangiogenic therapies in cancer. A phase 1 study is ongoing and preliminary results show that the ACE-041 is generally well tolerated when administered subcutaneously every three weeks. 4 out of 25 patients display stable disease lasting at least 6 cycles after previously having progressed on prior therapies (Bendell. et al., 2011). Our results suggest that this could be an effective treatment modality and we have not recorded any signs of development of resistance although this should be assessed using longer treatment periods. However, a word of caution is warranted. As our mechanistic studies revealed the surprising synergistic effect on angiogenesis by the combination of TGF- β and BMP-9 signaling it will be important to profile the ligand and receptor status of each patient ensuring the predicted treatment response.

4 FUTURE PERSPECTIVES

The first chemotherapeutic agent, a primitive alkylating compound, was presented in 1943. During the subsequent 15 years several of the compound classes we have today were developed. The first agents had some clinical benefit and remission in a subset of patients was achieved but patients often relapsed quite quickly. In the 1960s, the concept of cure by the use of chemotherapy started to emerge with the use of combination chemotherapeutic regimens (DeVita and Chu, 2008).

Targeted therapies have been developed and used in the clinic from the late 1990s, with a monoclonal antibody trastuzumab (Herceptin) being the first introduced followed by a receptor tyrosine kinase inhibitor imatinib (Gleevec) in 2001. In light of the very early lessons of treating cancer with drugs it is not entirely surprising that the new targeted therapies have not met the great expectations of increased overall survival. The patients develop resistance to these agents. We and others suggest that the combination of several different agents will prove more successful. To achieve this we need to develop a much bigger toolbox of targeted therapies.

To increase the targets, understanding of possible targets and the response to such therapies, more intricate studies are required. As an example, further characterization of the different CAFs populations and deeper understanding of the angiogenic process would most likely be highly rewarding. Many of the pathways identified today may have redundant capacity and possibly also display phenotypic changes that have to be investigated in detail. Crucial to these studies are the use of proper and complex model systems. Another important feature of targeted therapies will be the selection of the appropriate patients that will benefit from the therapy based on expression of different signaling molecules and/or receptors.

I find it probable that several pathways need to be targeted in conjunction to avoid resistance development and that these pathways need to be selected for each patient individually based on their specific molecular profile. This will pose huge problems for future clinical trials. The design of clinical trials must be altered radically for the introduction of new agents meant to be used in combinations on the market. Each component alone is likely to have only minor clinical efficacy on its own but together they can act in concert to produce clinical benefit.

5 ACKNOWLEDGEMENTS

This is where I finally can THANK everyone that has helped me on this journey, people who inspired me to go down this path and people who made it into a fantastic experience. I have learned many things, not just about science but also about life. Thanks to all of you for supporting, helping and guiding me through the winding path towards this thesis.

Kristian Pietras the best supervisor I could ever have gotten! Always present, in one way or another, to answer all imaginable questions. I can count the number of questions to which you haven't had an answer to for these years on one hand. Thank you for introducing me to the tumor microenvironment!

Ulf Eriksson the super co-supervisor, always enthusiastic with great wild plans, applicable to treating all various diseases, that always make me super excited about what I'm doing!

The Pietras girls: **Sara** always busy and very focused. Thanks for all long discussions "over" the mice in the lab, always answering my confused questions about TGF- β s and the best roomy during conferences. **Pernilla** another PDGF-C/CAF girl, so happy that you joined us! But I can't figure out how you juggle everything that is going on, can you teach me? **Eliane** the sweet Portuguese girl, good luck with your pericytes! I miss you **Marcela**!

The "uffe-puffe gruppe": I still consider myself as a part of your group. What would I have done before the other Pietras girls arrived without you?

Thank you **Monika** for all the fun and interesting late nights shared in the lab and on conferences. I have learnt so much from your very careful and patient approach to science. I'm so glad that you have stayed in our office not yet moving! **Carolina**, how quiet and boring life in the lab would have been without you! Thank you for everything, late night chats, sharing traveling tips, being an excellent tour guide and qPCR expert. Happy bubbly **Hong**, thanks for all laughs and discussions we have had together! **Erika**, the master organizer that always knows exactly how to get things done! Always cheerful **Annelie**, brightening up lunches with laughter. I'll always remember what you told me on June 15th last year, it helped a lot! **Ingrid**, tough but cheerful, organized and at the same time always with the best costumes for the parties and spex! **Xun**, thank you for all computer help, how would I have managed without it, I don't know and for sharing lots of Chinese sweets and customs. **Lars**, the black-metal bread making German, what a great mix. Pastry chef and baker **Sebastian**, you make every lab meeting to a joy! **Annika** always with a smile and super cute dresses. **Hanna**, good luck with PDGF-DD! Great to have, at least an undercover martial art girl, in the group. **Sofia**, I barely had the time to get to know you but it is great to have you here! **Michal** you followed us on the move to MBB, fun! **Xicong Liu**, welcome to the exciting world of research! **Daniel**, great to have you joining us! **Linda**, I know you are not an official part of "the uffe-puffe gruppe" anymore but you'll still end up under this section. Great to have you back again, although it was very inspiring to see you leave for you post-doc with the entire family!

Group of 4 or more...

It is the great mix of fantastic people, both past and present, and exciting research that contribute to the inspiring environment here! Thank you!

The “early bird“ CBZ:s: the first that struck me when we moved in with you was how early in the morning everything started, but that maybe says more of me than of you... **Christer**, it is always hard not to laugh at your songs and your razor sharp comments! They are so true. **Johanna** thanks for the mice and all good discussions! **Barbara** great office friend, always staying positive. **Radosa** it is fun to have you back again. **Guillem** the slightly grumpy Catalanian but there are smiles under there as well. Thanks **Maarja** for bringing the latest fashion to the lab, **Jen** for bringing the warm American spirit and **Konstantin** for patience explaining the world of confocaling for a beginner. Thanks **Sara, Helen, Jong Wook, Lwaki, miyuki and Peter** for adding to the joy. Thanks **Colin** for sharing my attitude towards order on the lab bench. **Kazuhiro, Ying and Annika** misses you all. **Maya**, can not say that I miss you because you are still around ☺!

The “Fuxe team”, Jonas, Joel and Mei-Fong with their long scientific discussions, always great to see your enthusiasm! **Tove, Jill and Maria**, I miss you all a lot!

The Björkegren group, I barely had the time to get to know you but it is great to have you here!

Lars, see you get you own group here as well. It’s been great sharing office. Good luck with your new group. Welcome **Yi**!

A big thanks to the **Matrix corridor** for contributing to the scientific and social environment at MBB!

Our MBB support team: Big thanks to **Theresa, Veronica, Pia and Emelie** for all the help in the animal facility, I couldn’t have done it without you, thank you!

Johanna, Jill, Sara, Chad, Torbjörn and Svante for helping and levelling all administrative and logistic hurdles.

Thanks to our Swedish and international connections! **Carl-Henrik Hedin** for all great TGF- β conferences and advises. **Peter ten Dijke, Lia Pardali, Marie-Jose Goumans and Lukas Hawinkels** for the TGF- β collaboration. **Helen Arthur and Zhenhua Zhai** in Newcastle and **Marta Páez-Ribes and Oriol Casanovas** in Barcelona for the collaboration on the endoglin project. **Glen Kristiansen, Holger Moch, Daniel Stiehl, Andrew Scott and Munro Neville** for the PDGF-CC collaboration

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A special thanks to my mentors and teachers that inspired me to take on this journey. Almost 15 years ago, after your talk **Pernilla Wittung-Stafshede** I decided to choose science. Thank you all **Tuula Jaalonon, Eri, Henrietta Hedin/Norman** that believed in me and though me all basic lab skills and also large scale drug development. I had a great time with you at AstraZeneca! I had a great summer with you **Louise**, you taught me so much! Thank you **Ola Hermansson** for you enthusiasm and giving me free hands with the stem cells! Last, but not least, **Kerrie**, for introducing me to flies and epigenetics and the whole American Midwest experience!

Thanks to **Patrick, Karolina and Sara** for making the course at Spetses to the greatest. **Magnus and Karin** for all fun and inspiring late nights discussions in Aspen.

Tack **Anna Ö**, numera W, för att du visade mig en för mig helt ny sport! Lycka till med allt i Umeå, jag blev så glad när jag hörde att det faktiskt går, även om det är lite synd att du och Fredrik inte kom tillbaka till Stockholm! **Elisabet** du ska veta hur mycket jag saknar dig på kontoret. Du spred alltid ett leende! Tack **Anna J** för att du lärde mig LCM, visade mig Umeå och även gjorde konferensen i Versailles mycket minnesvärd!

Jag tog glädjeskutt när ni äntligen flyttade hem igen **Lina och Patrik**! Det var otroligt roligt att kunna hälsa på er så långt borta men så mycket bättre nu när ni t.o.m. bor i Solna. Tack Lina för att du är en underbar vän som alltid stöttar och för allt roligt som vi har haft! De bästa minnena är från andra sidan jorden och jag kommer aldrig att glömma vår galna road-trip. Av nån anledning så är det alltid galet roligt när du är med! **Anna B**, jag förstod aldrig riktigt hur det gick till men jag är så glad att jag träffade dig på mattan. Saknar våra BJJ bravader nåt otroligt, aldrig var träningen så roligt som när du var med! Det går inte lika vilt till när vi träffas **Anna W** men det är alltid lika mysigt. Tack för alla idéer som vi bollat och mysiga middagar, bara så synd att det blir för sällan! **Elinor**, vi tappade bort varandra ett tag men när vi väl satt där på telefon så var det som om vi hade pratat för bara en vecka sen och prata timmar i telefon var vi alltid bra på! Det är så roligt att träffa dig igen. Another dear martial art friend **Kees**, so happy that we have kept in touch and it is always a pleasure to get to share you adventures all over the world! Tack **Markus**, bästa tränaren, för alla roliga träningstimmar, jag önskar att de hade blivit fler! Jag hoppas att du vet hur mycket de där träningarna betydde för mig och hur mycket jag saknar dem nu. A big thanks to my rock-climbing friends **Jamie and David** for all the fun I had in Iowa! Bonny, you and your family will always have a special place in my heart! Tack också till alla nya vänner **Johanna, Erik, Noomi, Gustav, Sophi m.fl.** som jag fått sen jag träffade Tobias och som alla lyckades få barn nästan samtidigt, underbart! **David och Reenu**, alltid lika roligt att träffa på några som också har ett stort matintresse eller tankar på att våga prova sina vingar någon annanstans! Hur hade det blivit om jag inte hade träffat **Jona** "the match maker" den där dan i lunchrummet på CMB? Tack Jona!

Det är jätteroligt att ha fått en jättestor familj helt plötsligt. Tack för att ni välkomnade mig med öppna armar, hjälper oss så mycket och för att jag får ha er Tobias! Ett speciellt tack för alla underbara upplevelser i Japan.

Mamma, tack för att du alltid varit min förebild, klippa och inspiratör! Genom dig har jag lärt mig att man alltid kan lära sig att göra vad som helst och att ingenting är omöjligt, bara man vill. Du är min stora förebild, jag hoppas att jag kan bli, en iallafall nästan lika bra, mamma själv! Underbara lillasyster **Tina**! Ledsen, du kommer alltid att vara lillasyster ☺ men också min bästa vän som jag alltid kan vända mig till. Du har lärt mig jättemycket och utan dig hade jag nog aldrig vågat upptäcka fightingen. Jag är så stolt över dig, även om det svider lite att du nu fick blått och inte jag ☺.

Xander världens underbaraste lilla kille, full med skratt och energi som smittar av sig!
Vi väntar på att se vad du är för en filur "**lillskrutt**".

*Älskade, underbara **Tobias**, tack för du alltid finns där för mig och stöttar mig i det jag vill göra. Du får mig att inse vad som är viktigast här i livet, du, **Xander och lillskrutt** är det värdefullaste jag har!*

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